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(54) Title: METHOD FOR DIAGNOSING A PATIENT FOR CHLAMYDIA

(57) Abstract

A method of detecting chlamydia in an extracellular sample is provided which comprises contacting the sample with an idiotypic antibody to GLXA to form an immunocomplex and detecting the immunocomplex.

METHOD FOR DIAGNOSING A PATIENT FOR CHLAMYDIA

REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of application Serial Number 08/034,572, filed March 19, 1993.

BACKGROUND OF THE INVENTION

This invention relates to a method for diagnosing a patient for the presence of Chlamydia.

Chlamydial infection is a diverse group of conjunctival, genital, respiratory, and neonatal infections occurring primarily on mucosal surfaces. The etiologic agent of the infection is an obligate intracellular bacterial parasite of eukaryotic cells, chlamydiae. There are four genetically different species in this genus, with certain similarities in morphology, intracellular developmental cycle and antigenic responses: Chlamydia trachomatis, Chlamydia psittaci, Chlamydia pneumoniae. and Chlamydia Percorum. The infection by C.trachomatis is limited to humans. Fifteen serovars are differentiated based on the antigenic variations of the major outer membrane protein (MOMP) (Grayston and Wang,J. Infect. Dis., 132:87, 1975). Serotypes D-K, are the most common cause of sexually transmitted venereal diseases. Conservatively, more than 4 million cases of chlamydial sexual infections occur each year in the United States making it more prevalent than all other sexually transmitted diseases combined. The diseases include nongonococcal urethritis, mucopurulent cervicitis, acute epididymitis, ectopic pregnancy and pelvic inflammatory disease (PID, endometritis, salpingitis, parametritis and/or peritonitis). The infection in women can be quite damaging: Among 250,000 cases of pelvic inflammation diseases caused by this organism in the U. S. each year, 10% lead to infertility. When infants are born to chlamydia-infected mothers, they are at high risk of developing inclusion conjunctivitis and pneumonia. C. trachomatis serovars A, B, Ba, and C cause trachoma, an infection of conjunctival epithelial cells. The chronic and secondary infections induce the infiltration of subepithelial lymphocytes, forming follicles and the invasion of fibroblasts and blood vessels to the cornea, leading to blindness. On the other hand, the formation of the scar and malformation of the eyelid, causing trichiasis' constant scraping of the cornea by the eyelash can also

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lead to corneal opacification and blindness. There are approximately 500 million trachoma cases in the world, and between 7 and 9 million are now blind because of its complications making it the world's leading cause of preventable blindness. The prevalence of active trachoma is high in early age. There are 80 million children in need of treatment. It has been an enormously important health problem in the Middle East, North Africa, South Asia and North India.

C. psittaci mainly affects animals and birds. It had, and still has a great economic impact in dairy, wool and meat industries. There are 9 serovars from mammalian species, 7 serovars from avian species and 2 biovars from koala bears. Mammalian serovar 1, 2, 3, and 9 infect cattle and sheep, causing a wide range of disorders from placenta and fetus infection and other reproductive problems, including polyarthritis-polysistitis, encephalomyelitis, conjunctivitis as well as intestinal infections. Although numerous attempts have been made to produce vaccines, only modest success has been achieved (Schnorr, J. Am. Vet. Med. Assoc. 195:1548, 1989). Serovars 4, 5, and 6 are the causes of abortions, pneumonia and polyarthritis in porcine species. Serovar 7 represents chlamydial strains of feline conjunctivitis, rhinitis and pneumonitis and serovar 8 includes guinea pig inclusion conjunctivitis. The avian strains often cause human infection in bird handlers and poultry processing workers.

C. pneumoniae is a newly identified species. To date, one serovar has been identified, TWAR (Grayston, Proceedings of the Seventh International Symposium on Human Chlamydial Infections, Pg. 89, 1990). Current evidence suggests that C. pneumoniae is a primary human pathogen that is transmitted from human to human and causes about 10-20% of community acquired pneumonia in adults. It has become the main causative agent of human respiratory diseases such as pneumonia, bronchitis, pharyngitis, and sinusitis and a possible agent in reactive arthritis. Epidemics have occurred in hospitals, in the military and families. The serological finding from many countries have shown that 50-55% of adults with antibody against TWAR antigen are specific for C. pnueun-

oniae. It is the major bacterial cause of illness in newborn. The infection to elderly persons and those with chronic diseases may cause serious illness or even death.

Chlamydia pecorum was identified as a new species in 1992. It is prevalent in cattle, sheep and swine and a number of exotic birds and invertebrates. In cattle it causes encephalomyelitis, upper respiratory disease and pneumonia. Whereas in sheep, it has been isolated in lambs with polyarthristis. It is widespread and pathogenicity in animals has still not been determined for some species (Storz et al, Proceedings of the Eight International Symposium on Human Chlamydia Infections, Pg 563, 1994).

The pathogenicity of chlamydial infection is not well understood. It is long known that different individuals infected by these serovars exhibit different clinical manifestations. It has been proposed that it was likely due to the variation of the host immune response. It has been shown that immunologic response to the synthetic Th/B cell epitopes in the various inbred strains of mice is different, indicating that the T helper epitope is recognized in the context of the multiple major histocompatibility complex.

The target of chlamydial invasion are typically epithelial cells of a host. It is still not certain how the chlamydial elementary bodies (EB), (a sporelike, spherical particle, about 300 nm in diameter), enter the host cell: receptor-mediated endocytosis, and/or non-specific high affinity absorption. It has been reported that two proteins, 18 and 32 kD of C. trachomatis bind to Hela 299 cell membrane preparations. Recently, another heat-liable protein membrane protein, 38kD, was proposed as binding to Hela cell line, suggesting a ligand like mechanism. It has also been proposed that since chlamydia have the ability to infect a wide variety of mammalian cells in vitro, there must be some adherence mechanism for the establishment of the infection. The major outer membrane protein was proposed as such an adhesin. Recently, it has been demonstrated that a heparin sulfate-like glycosaminoglycans present on the surface of

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chlamydia organisms is required for attachment to host cells. The receptors on
the host cells have also been studied. It was suggested that proteins, 18,000 and
31,000 kD from Hela cells are the receptors due to trypsin sensitivity for the EB
specific binding. It also has been shown that C. trachomatis and C. pneumoniae
bind specifically to a lipid on Hela cells. Nuclear magnetic resonance spectros-
copy analysis and atom bombardment mass spectrometry show that it was
phosphatidylethanolamine (PE). At the same time ganglia-series glycolipids were
found specifically bound to EBs. All those findings suggest that the mechanism of
endocytosis by epithelial host cells is still a matter of uncertainty. Once the EBs
enter the host cells by endocytosis, depending upon conditions, they are trans-
formed into a metabolically active, non-infectious reticulate body (RB). The prime
purpose of RBs is intracellular replication by binary fission using host
metabolites. This occurs in a membrane-bounded vesicle, termed an inclusion.
This inclusion (endosome) can resist the fusion with the lysosomes of host cells.
Each RB eventually gives rise to one or more EBs which can initiate another
infectious cycle. Host cells may be lysed by release of inclusion bodies or
undamaged by exclusion body excytosis. Surface antigens are thought to direct
both phagocytosis and evasion of phagolysosomal fusion.

Surface components of chlamydia actively interact with host cells and with
the host's immune system. They are believed to account for the attachment,
endocytosis and the immune response, but the exact nature and regulation of
these interactions has not yet been fully identified. Several distinct antigenic
components of C. trachomatis, C. psittaci and C. pneumonia have been in-
vestigated including the identification, characterization and function in chlamydial
infection. Moreover, it is of importance to determine the mechanism of infection
and determine the protective antigens. Surface exposed antigens are the main
targets of much research since they are accessible to the immune or other
defense systems. The antigens most actively investigated include major outer
membrane proteins (MOMP) chlamydial lipopolysaccharide, 60-kD heat shock

protein (.HSPO 60) adhesins and a glycolipid exoantigen termed the exoantigen (GLXA).

In the outer membrane of chlamydia there are three cysteine-rich proteins 57, 40, and 12.5 kD which resembles the matrix proteins of gram-negative bacteria. The 57 and 12.5 kD proteins can not be found in the replicating form of the bacteria RBs. As the major outer membrane protein (MOMP), 40 kD, is abundant in both infectious EBs and RBs. In RBs, the protein could function as pore-forming proteins that permit exchange of nutrients for the reticulate bodies. Genetic and molecular characterization have shown that this protein is composed of four variable segments (VS) interspersed among five constant segments. Those variable segments are surface exposed and have the determinants of serovar, subspecies and species specificity.

The studies on immune responses to this protein are mostly carried out by immunization of animals with purified protein. In vitro neutralization experiments have been conducted using the mixture of poly or monoclonal antibodies specific to MOMP and EBs to infect cell culture. These experiments indicate that the antibodies specific to MOMP protein or one single epitope prevent the inclusion bodies formation in cell culture. The mechanism of the neutralization does not involve inhibition of the attachment or penetration, but rather interfere with the process after internalization. Using monoclonal antibodies generated by the whole elementary bodies of serovar B, the monoclonal antibodies which recognized the immune accessible MOMP epitope in dot blot assays, neutralized the infectivity of organisms of monkey eyes. The protection was serovar-specific. In a later experiment by using Fab fragments of the monoclonal antibody, it has been further demonstrated that monovalent Fab neutralized the infection by preventing the attachment to Syrian hamster kidney (HaK) cells. Confirming that the protection is not due to the aggregation of bivalent IgGs. T cell epitopes of MOMP have also been investigated. T cell proliferation responses were found in splenic T cells obtained from A/J mice immunized with MOMP in the presence of

overlapping synthetic peptides which represent primary sequence of serovar A MOMP. The synthetic peptides which produced T cell response correspond to surface-accessible serovar-specific epitopes located in variable domains (VD) VD I and VD IV. By using a similar approach, it was found in BALB/cByJ mice that VDIII fragment is T cell dependent. It also has been shown that by using chimeric T/B cell peptides derived from two epitopes of MOMP, one is a conserved T helper cell epitope and the other is a serovar A specific neutralizing epitope. Some mice immunized with this peptide produced high-titered serum-neutralizing antibodies, while others did not. Although MOMP has been a most intensively studied surface antigen and the neutralization antisera has been produced in experimental animals, there are still many unsolved questions regarding the immune response. For example, the neutralization of infection is serovar specific, thus, it is limited as a vaccine candidate. The neutralization is still limited to in vitro studies, and there has been no convincing in vivo protection from challenge by immunization with any MOMP or chimeric epitopes known.

It has long been known that a chlamydia genus specific antigen was a glycolipid (Dhir et al, J. Immunol. 109: 116-122, 1972). Much later, it was found that lipopolysaccharide (LPS) was in the outer membrane of both EBs and RBs of chlamydia. It has a chemical structure similar to enterobacterial LPS of the Re chemotype (Nurminen et al, Science, 220: 1279-1281, 1983). The monoclonal antibodies prepared by immunization with EBs of serovar L2 were specifically against LPS of chlamydia, but not LPS from N. Gonorrhoea, S. typhimurium, or E. coli. However, antibodies produced by S. typhimurium Re LPS or lipid A recognized chlamydial LPS (Caldwell and Hitchcock, Infect. Immun., 44: 306, 1984). This demonstrated that chlamydial LPS has an unique antigenic domain compared to other gram-negative bacteria. Further characterization has shown that a chlamydia-specific domain contained in its saccharide portion, 3-deoxy-D-manno-2-octulosonic acid (KDO) with a sequence of αkDO (2-8)- αkDO(2-4) α-kDO(kDO$_3$). The 2.8 linked moiety is the structural characteristic of chlamydial

LPS. Studies have also been carried out in the distribution and the relocation of LPS on the outer membrane during the developmental cycle. By immunostaining with a monoclonal antibody it was shown that LPS is loosely bound in the membrane during the developmental cycle, and not shed into media.

Chlamydial LPS was thought to be an ideal antigen for the vaccine candidate because of its abundance on the surface and its being antigenic. LPS was suspected as an important virulent determinant in the early steps of the infection and the antibodies specific to it serve some function in resolving the chlamydial infection. However, little is known concerning the biological function of LPS or the immunological response to it. It appears that the antibodies which are specific to LPS only have been useful in the diagnosis of chlamydial infection and location of LPS, but not effective in resolving an infection.

Other genus-specific chlamydial antigens are 57 to 60 and 75 kD proteins which have been identified as related to the heat shock protein (HSP) family. This was done by comparing the sequence of the operons encoding these proteins to the groE stress response operon of E. coli or B. megaterium. The antigenic identity of 57 kD protein was confirmed by the reaction with anti-HSP-60 antiserum. The 60 kD protein elicited an ocular hypersensitivity response in immune guinea pigs, which was characterized by a predominantly mononuclear macrophage and lymphocyte cellular infiltration (Watkins, et al, Proc. Natl. Acad. Sci. USA 83: 7580, 1986, Morrison et al, S. Exp. Med. 169: 663, 1989). This was the first indication that an antigen is responsible for delayed hypersensitivity in chlamydial ocular infection. The precise involvement of this protein in stimulating immunopathogenic responses in human chlamydial diseases has not been determined. There is evidence that shows a certain percentage of sera taken from women with PID, ectopic pregnancy and tubal infertility have high anti-chlamydial antibodies reacting to chlamydial HSPO-60 heat-shock protein. However, not every patient serum which has high titer to chlamydia reacts with it, indicating that either HSP-60 is not surface exposed or antigenicity is MHC

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restricted.

The 75-kD protein was found preferentially transcribed during heat stress of chlamydial organisms. The monospecific antibodies from rabbits raised against 75-kD protein were found to bind to the organism and neutralized the infection in vitro. It is an exposed antigen in the outer membrane.

Genus-specific glycolipid exoantigen (GLXA) was originally isolated from the supernatants of chlamydia infected cell cultures (Stuart and MacDonald, Current Microbiology, 11: 123, 1982). it has been characterized chemically, biologically and serologically in recent years (Stuart and MacDonald, Proceedings of the Sixth International Symposium on Human Chlamydia Infections, p167, 1986, Stuart et al, Immunology, 67: 527, 1987). Mass spectrographic analysis indicated that GLXA contains polysaccharides: gulose, (not glucose), mannose and possibly galactose, while the lipid component has fatty acids of chain length C17 and C18:1. There is no KDO or lipid A found in its structure. It is produced and released from the infected cells during the growth cycle in vitro. Transmission electron microscopy utilizing colloidal gold-conjugated goat anti-mouse second antibody to detect the specifically bound monoclonal antibody revealed that GLXA is mostly extracellular 48 hours after the infection (Stuart et al, Immunology, 74: 747, 1991) which is different from that found for chlamydial LPS. Human sera from patients with clinically defined lymphogranuloma venereum (LGV) contain IgG antibodies which recognize GLXA (Stuart and MacDonald, Immunology, 68: 469, 1989), demonstrating the immunoaccessibility in the natural infection. But, there was little information on its function in the chlamydial infection and the immune response to it. The overall immunological reaction to chlamydial antigens is not well understood. It is still not known how the chlamydia evade the host immune surveillance. Antibodies found specific to chlamydial antigens in infected human patients have shown little protection for later infection. Although chlamydia mainly affects mucosal surfaces, the clinical relevance of the IgA immunity to it has not been completely described. The

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feasibility of chlamydia vaccine depends on producing a protective host defense which may include S-IgA response, a cell mediated response and possibly a humoral antibody. In addition, the ability to produce large quantities of this antigen indicates a synthetic and/or chimeric antigen may be the method of choice.

Idiotypes have been intensively studied following Jerne's network theory in 1974. One of his major proposals is the self-regulation of the immune system through a network of idiotype-anti-idiotype interactions (Jerne, 1974). It is suggested that the idiotopes on a single antibody molecule can mimic (that is, be the "internal image") of any foreign or self epitope at the molecular level.

All idiotypes of a single immunoglobulin molecule have been found to be located on Fv (fragment variable) region by studies showing that the inhibition of binding of anti-idotypic antibodies to the idiotype is the same between Fv and Fab(Givol, 1991). In general, anti-idotypic antibodies are divided into three types $Ab_2 \alpha$, $Ab_2 \beta$ and $Ab_2\varepsilon$. Only $Ab_2 \beta$ binds to the complementary determining region, thus can be the internal image of the antigen. The occurrence of Ab_2 displaying internal image of properties must adhere to the following criteria; (1) binding onto Ab_1 and to any other anti-nominal antigen antibodies from another species and lack of reactivity with Ab_2 to other antibodies; (2) inhibition of the binding of Ab_1 to the specific antigen, the nominal antigen, and (3) the ability to elicit the synthesis of Ab_3 with anti-antigen specificity in animals without previous exposure to the antigen (Ertl and Bona, 1988).

The important role of anti-idiotypic antibodies in vivo has been shown in numerous experiments. The administration of anti-idiotypic antibodies was found to elicit different effects: either suppression or enhancement of the responses to the specific idiotype(Hart, 1972, Kennedy, 1983). In autoimmunity, it certainly plays an important role. The pathology associated with many autoimmune diseases is most likely due to (at least in part), a direct idiotype-anti-idiotype interaction of the autoimmune antibodies with anti-idiotypic antibodies. Idiotypic

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specificity in a specific antibody were first characterized, by demonstrating that specific hapten binding could inhibit idiotype recognition. The first experimental support for the validity of the internal image was presented by Sege and Peterson in 1978 by using anti-idiotype as a probe to identify cell surface receptors.

The best information for the exact molecular basis for the mimicking presently is obtained from the X-ray crystallography of the idiotype-anti-idiotype complex. The basis of molecular mimicry of the antibodies can be either local sequence homology to the original protein as in a reovirus system or, in most cases, identical conformations from entirely different amino acid sequences as in the hemoglobin-myoglobin family of proteins. X-ray crystallography and sequence data in the later studies showed that identical, functional conformations can be assumed by proteins that differ by as many as 137 of 141 amino acids. The studies of the crystal structure of idiotope-anti-idiotope complex in the anti-lysozyme antibody and the anti-idiotope have demonstrated that a private idiotope consists of 13 amino-acid residues, most from the complementarity-determining regions, but including three residues from the third framework region of its VL domain. Seven of these residues are common with the paratope of anti-lysozyme antibody, indicating a significant overlap between idiotope and antigen-combining site. Idiotype has been a unique tool in characterization and manipu-lation of the immune response since it was found and realized: as a clonal marker to follow B cell development, somatic mutation and fate of clones of B cells. They have been used as a phenotypic marker for germ-line V genes. Anti-idiotypic antibodies which bear the internal image of external pathogens such as virus, bacteria or parasites have been used as surrogate antigens for vaccine and are being used in treating B cell lymphoma and autoimmune disease such as encephalomyelitis. In addition, it has been shown that anti-idiotypic antibodies can induce T-cell responses in which either by toxic T-cells or T-helper cells are produced which recognizes the original antigen.

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Since chlamydia are intracellular parasites, current tests emphasize the importance of collecting cellular material from the specimen to be tested. When utilizing these tests, an extraction method is required in order to separate the chlamydial antigen from the cellular material when an immunological test is performed. Thus, presently available diagnostic tests for chlamydia are based upon detecting the presence of major outer membrane protein (MOMP) or lipopolysaccharide (LPS). The presently available diagnostic procedures for MOMP are undesirable since they lack sensitivity and specificity. Diagnostic tests based upon LPS are undesirable since LPS is present only in small amounts and the procedures needed to concentrate LPS are complex. In addition, diagnostic tests based upon cellular material are undesirable since they require culturing of cells which is time consuming and they require processing of cellular material which is time consuming and complex.

It has also been proposed in Current Microbiology, Vol. 28, pages 85-90, 1994 to provide a diagnostic test for cells infected with chlamydia based upon the production of GLXA from cultured cells infected with chlamydia. While the disclosed assay method for GLXA was shown to be useful for assaying for GLXA obtained from cell cultures, no determination has been made that the assay method is useful for assaying GLXA in bodily fluids such as urine or serum.

While the diagnosis for the presence of GLXA antibodies in a bodily fluid is useful to determine whether an animal or a human has been previously infected with chlamydia, it is not useful to determine whether the animal or human is infected with chlamydia at the time of diagnosis.

Accordingly, it would be desirable to provide a diagnostic test for chlamydia which is specific and sensitive. In addition, it would be desirable to provide such a diagnostic test which is suitable for all serovars of chlamydia. Furthermore, it would be desirable to provide such a test which is specific and sensitive to an antigen of chlamydia which can be found in extracellular biological fluids of animals and humans which does not require lysing of cells. Such a test

would eliminate the need to culture or otherwise process cells.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a binding curve of guinea pig antisera to monoclonal GLXA-Ab_1.

Figure 2 is a binding curve of guinea pig anti-monoclonal GLXA-Ab_1 IgG antisera to normal mouse IgG before and after immunosorption.

Figure 3 is a curve showing the inhibition of the binding of monoclonal GLXA-Ab_1 to GLXA by absorbed guinea pig anti-idiotypic antisera.

Figure 4 is a curve showing fractionation of guinea pig anti-idiotypic IgG.

Figure 5 is a curve showing the inhibition of the binding of monoclonal GLXA-AB_1 to GLXA by guinea pig anti-idiotypic isotypes.

Figure 6 is a curve showing the binding of rabbit anti-anti-idiotypic antibody to guinea pig anti-idiotypic IgG.

Figure 7 is a curve showing the inhibition of the binding of monoclonal GLXA-Ab_1 to GLXA by rabbit GLXA-Ab_3.

Figure 8 is a curve showing the detection of chlamydial specific ribosomal RNA from primates.

Figure 9 is a curve showing the effect of GLXA-Ab_3 IgG on ocular infection by clinical disease score.

Figure 10 is a curve showing the inhibition of binding of monoclonal GLXA Ab_1, to GLXA by a hybridoma clone.

Figure 11 is a curve showing the direct binding of chlamydia patient antiserum to monoclonal GLXA-M Ab_2.

Figure 12 is a curve showing that rabbit anti-chlamydia antiserum recognizes monoclonal GLXA-M Ab_2.

Figure 13 is a curve showing the protection of mice from chlamydial infection by immunization with monoclonal GLXA-M Ab_2.

Figure 14 is a protection curve by monoclonal GLXA-M Ab_2 IgG after a high dose of ocular infections.

Figure 15 is a curve showing the effect of alum on the protection of mouse chlamydial infection.

Figures 16 A and B show curves of the time course of ocular infectivity after immunization with monoclonal GLXA-Ab$_2$.

SUMMARY OF THE INVENTION

The present invention is based upon the discovery that a diagnostic test for the presence of chlamydia in a human or an animal can be effected with a extracellular biological fluid of the human or animal such as urine, tears, saliva, nasal phargynial fluid, cervical swab fluid or vaginal swab fluid. The extracellular biological fluid is tested for the presence of GLXA with the idiotypic antibody to GLXA (hereinafter GLXA-Ab$_1$) or the anti-anti-idiotypic antibody (hereinafter GLXA-Ab$_3$) which is the antibody to the anti-idiotypic antibody to GLXA-Ab$_1$ (hereinafter GLXA-Ab$_2$). In accordance with this invention, it has been found that an extracellular biological fluid obtained directly from the human or animal can be assayed for the presence of GLXA which is a sensitive and specific marker for all known forms of chlamydia. The diagnostic use of this invention can be utilized in any of a variety of modes including direct assay,indirect assay, competition assay or sandwich assay. Thus, the diagnostic test of this invention provides substantial advantages over prior art tests which require cellular material from the human or animal being tested.

DESCRIPTION OF SPECIFIC EMBODIMENTS

The GLXA is obtained and purified by any presently available method. Thus GLXA can be isolated on an octylsepharose column and eluted with alcohol or isolated on DEAE Sepharose and eluted in low pH aqueous solution or isolated on a polyacrylamide bead column and eluted with low pH aqueous solution of KSCN. (5M). In a preferred method GLXA is obtained and purified from supernatant of infected cell cultures by presently available methods such as by exclusion chromatography over a Sepharose 6B-Cl column in 0.075 M phosphate, 0.154 M NaCl, pH 7.2. The GLXA appears in the front fraction of the

column and is detected by a chemiluminescence assay using an acridinium ester conjugated monoclonal GLXA-Ab$_1$. The fractions containing the GLXA are usually contaminated by nucleic acids, but not protein. The GLXA fractions are pooled, concentrated and treated with RNase and DNAase at pH 8.0 for about 2 hours at 37° C. The mixture is rechromatographed over the same column and is now pure. It can be stored at 4° C (with or without preservative).

The antibody can be polyclonal or monoclonal. In the production of monoclonal antibody, an idiotypic antibody GLXA-Ab$_1$ is provided by immunizing an animal, usually a mouse, with GLXA or the chlamydia bacteria as the antigen. Immune spleen cells of the animal then are identified, isolated and fused with lymphoma or myeloma cells by being contacted with a fusing agent such as polyethylene glycol such as by the procedure of Kohler & Milstein, Nature 256: 459, 1975. The fused cells then are incubated in a selective medium such as HAT medium which precludes the growth of unfused malignant cells. The hybridoma cells are cloned by limiting dilution and supernatants are assayed for secreted monoclonal antibody of desired specificity. A suitable hybridoma for producing GLXA-Ab$_1$ is deposited in the American Type Culture Collection and identified as ATCC H.B.11300. Monoclonal antibodies also can be obtained by ascitic growth of hybridomas in vivo. Alternatively, the lymphocyte cells can be immortalized by exposure to Epstein-Barr virus. The idiotype antibody, GLXA-Ab$_1$ is useful in producing GLXA-Ab$_2$ which, surprisingly is active in immunizing against or neutralizing a chlamydia infection. In addition, GLXA-Ab$_2$ is not species specific but is genus specific in that its immunization and neutralization activity is useful in many animal species. The monoclonal GLXA-Ab$_2$ also can be produced by the process utilizing hybridomas set forth above for GLXA-Ab$_1$ but by utilizing GLXA-Ab$_1$ as the antigen. A suitable hybridoma for producing GLXA-Ab$_2$ is deposited in the American Type Culture Collection and identified as ATCC H.B.11301. GLXA-Ab$_2$ also can be produced as a polyclonal antibody and, as a polyclonal antibody it is active for immunization against or neutralization of

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infection by chlamydia. Polyclonal antibodies are made in any conventional manner wherein a first animal is immunized with GLXA as the antigen and polyclonal GLXA-Ab$_1$ is isolated from the animal's serum. The polyclonal GLXA-Ab$_1$ or monoclonal GLXA-Ab$_1$, then is injected into a second animal of a different species from the first animal and polyclonal GLXA-Ab$_2$ then is recovered from the second animal's serum.

Any conventional test procedure can be utilized in the present invention including radioimmunoassay, enzyme-linked immunosorbent assay, fluorescent assay, chemiluminescent assay, competitive immunoassay, membrane-based immunoassay, precipitation, agglutination, antigen capture or the like.

While the present invention can be utilized to directly test extracellular biological fluids, it is to be understood that the present invention also is useful for testing for chlamydia in cells obtained from a human or animal wherein the presence of GLXA can be determined. However, it is preferred to test an extracellular biological fluid since the manipulative steps needed to effect the assay are less complex than with samples having cells which are lysed.

The labeled antibody Ab$_1$ or Ab$_3$ or Fab fragment thereof can be utilized to interact directly with the extracellular biological sample to form a bound complex with any GLXA present in the sample. The concentration of GLXA present in the sample is determined by measuring the concentration of the detectable label in the antigen (GLXA)-antibody complex formed, if any. Alternatively, the concentration of GLXA can be determined with a conventional competitive assay wherein the Ab$_1$ or Ab$_3$ -based antibody can interact either with an unknown concentration of GLXA and a known concentration of Ab$_2$ or Fab fragment of Ab$_2$ The concentration of GLXA in a sample also can be determined indirectly with labeled Ab$_2$ or a Fab fragment of Ab$_2$. In this procedure, any GLXA present is first complexed with Ab$_1$ or Ab$_3$ of Fab fragment thereof. The labeled Ab$_2$ or Fab fragment thereof then is interacted with the complex to form a second complex containing the detectable label.

A preferred diagnostic procedure is:

Direct Sandwich Assay

Where immobilized Ab_1 or Ab_3 or Fab fragment thereof captures any GLXA present in the sample of extracellular biological fluid. The concentration of bound GLXA is then determined by interaction of labeled Ab_1 or Ab_3 or Fab fragment thereof and measurement of the detectable label in the antigen (GLXA)-antibody complex formed, if any.

The following examples illustrate the present invention and are not intended to limit the same.

Example 1

Throughout this study, two serovars of C. trachomatis were used. Serovar B (Strain Har 36) organisms originally isolated from conjunctival scraping of a child, were grown at 37° C on McCoy cell monolayer with Eagle's Minimum Essential Medium with Hank's balanced salt solution (HMEM, Whittaker, MD) and stored at -70° C in the same medium with 10% of dimethyl sulfoxide (Sigma Chemical Co., MO). Serovar C (TW-3) was grown in mass tissue culture in McCoy cells. The elementary bodies were purified by centrifugation through renograffin and resuspended in phosphate-buffered saline and stored at -70° C.

Chlamydia free Hartley strain inbred guinea pigs were originally obtained from Jackson Laboratory (Bar Harbor, ME), bred and maintained in an animal facility. One year old females were used in this study.

Female New Zealand rabbits free of Pasteurella were obtained from Mill Broock Farm (Hadly, MA). The rabbits were about 6-8 pounds when received and used within one week. BALB/c ByJ (H-2d) mice were obtained from Jackson Laboratory (Bar Harbor, ME). Young adult cynomolgus monkeys (Macacca fasicularis) were obtained from Charles River Primates, Inc. (Port Washington, NY) which were free of clinical ocular diseases. All the primates had been used for a previous ocular infection with C. trachomatis serovar C elementary bodies, some primates had been previously immunized with a chlamydial protein. The

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primates challenged at this study, were free of the disease and were not immune. They were randominized prior to the initiation of the experiment. All procedures were performed under anesthesia.

Production of Polyclonal Anti-idiotypic Antibodies

For the production of polyclonal anti-idiotypic antibodies, each of six guinea pigs was immunized subcutaneously with 150 ug of monoclonal GLXA-Ab_1 (89MS30) IgG in 1 ml of H_2O plus 1 ml Maalox Plus suspension (William H. Roger, Inc., PA). Three weeks later the guinea pigs were boosted with 100 ug of monoclonal GLXA-Ab_1 in 1 ml of Maalox (aluminum hydroxide, alum) and H_2O, and were boosted on a monthly basis.

For the production of anti-anti-idiotypic antibodies, three rabbits were immunized with absorbed anti-idiotypic guinea pig isotype IgG-I, 300 ug in 1 ml of alum and 1 ml of H_2O intradermally on multiple sites and boosted three weeks later using the identical protocol. Subsequently, rabbits were boosted at monthly intervals. For the production of monoclonal anti-idiotypic antibody, GLXA-Ab_2 (91 MS441), fifty ug of KLH conjugated monoclonal GLXA-Ab_1 IgG was injected into five 9-week old female BALB/cByJ mice intraperitoneally in an equal amount of complete Freund's adjuvant (Sigma, MO). The same amount was used in the subsequent booster at day 14 and later on a weekly basis for five weeks in the presence of incomplete Freund adjuvant. A final booster injection was given 3 days before the removal of the spleen.

Monoclonal GLXA-Ab_1 IgG was isolated by protein A affinity chromatography. The ascites which contains monoclonal GLXA-Ab_1 IgG was first centrifuged 2000 rpm for 10 minutes at 4° C and passed through glass wool to remove lipids and precipitates. The affinity column (1.5 x 9 cm) packed with 2 ml Protein A Sepharose CL4B (ZYMED, CA) was first equilibrated in a binding buffer (1.5M glycine, 3M NaCl, pH 8.9) (Jackson ImmunoResearch Laboratories, PA). Two ml ascites diluted with equal amount of binding buffer was loaded onto the column at 0.6-0.8 ml/minute. The column was stopped for about 30 minutes to allow

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binding and then rinsed with about three bed volumes of binding buffer. The bound IgG was eluted with 0.1 M citric acid, pH 3, into glass tubes which contained 50 ul 0.1 M TBS, pH 8.0 to equilibrate the pH. IgG was detected by an absorption at 280 nm. The absorption larger than 0.1 was pooled,and dialyzed against 0.075 M PBS, pH 7.2 overnight. The purity of the IgG isolated was confirmed by SDS PAGE on a PhastSystem (Phamacia, NJ).

The specific response of guinea pig immunized with monoclonal Ab_1 IgG was detected by direct enzyme-linked immunosorbent assay. A 96 well plate with Immulon 2 removable strips (Dynatech, RI) was coated with 0.4 ug of monoclonal Ab_1 IgG per well in coating buffer (0.015M $NaHCO_3$, 0.3M glycine, 0.02% NaN_3 with 0.06M polyethylene glycol, PEG), pH 9.6. The plate was stored at 4° C overnight. Each plate was rinsed three times with 0.05% Tween 20 in 0.075M PBS and blocked with 1% BSA/PBS for 2 hours at room temperature. Again, the plate was rinsed three times. The pre-immune sera or antisera from guinea pigs were serially diluted with 0.075M PBS and 100 ul of each was added to each well in duplicate. The mixture was incubated at room temperature for 1 hour and rinsed. Goat anti-guinea pig IgG (H & L) horseradish peroxidase conjugate (Jackson ImmunoResearch Labs, Inc. PA) 1:1000 was added, incubated for one hour and rinsed. TMB substrate (Kirkegaard and Perry Laboratories, MD) was added after rinsing. The absorbance at 405 nm was determined using a Vmax microwell reader (Molecular Devices Corp., CA). The antisera from rabbits immunized with guinea pig IgG-1 was assayed in the similar manner. Each plate was coated with guinea pig IgG-1 and the second antibody was goat anti-rabbit (H and L) horseradish peroxidase conjugate (Jackson Immune Research Labs. Inc., PA).

Normal mouse IgG (Jackson ImmunoResearch Labs Inc., PA) was conjugated to Affi-Gel I0 (Bio-Rad Laboratories, CA) as indicated by the manufacturer. Briefly, 5 ml Affi-Gel I0 slurry was transferred to a glass fitted funnel connected to an aspirator and washed with three bed volumes of isopropyl

alcohol followed by three bed volumes of ice cold deionized water. Normal
mouse IgG (5 mg/ml), 10 ml was mixed with Affi-Gel 10 in a vial. The coupling
was done at 4° C overnight with gentle end-to-end agitation. A column (0.9 x
5ml) was packed with the coupled gel and rinsed with 0.075M PBS, pH 7.2. The
UV absorbance at 280 nm of the effluent was monitored. The highest absor-
bance of this portion was used to test for protein content by the Bradford Assay
(Bio-Rad Laboratories, CA) to evaluate the conjugation.

A pool of one guinea pig antisera 3, 4 and 5 weeks after the immunization
was absorbed by affinity chromatography on a normal mouse IgG-agarose
column. The column was prepared as above. The antisera, about one void
volume, was loaded onto the column and incubated for 30 minutes at 4° C and
eluted with 0.075M PBS, pH 7.2. The antiserum was absorbed a second time
with a freshly prepared column.

Five ml of guinea pig antisera which had been absorbed by normal mouse
IgG, was loaded onto a protein A conjugated sepharose column, and rinsed with
0.02M phosphate-citrate buffer, pH 7.3. Guinea pig immunoglobulin subclasses
IgG-1 and IgG-2 were eluted separately using a step pH gradient of 4.9 until no
protein was detected in the effluent. The column was then eluted with a low pH
gradient, 4.3. After dialysis against 0.02 M phosphate-citrate buffer, pH 7.3, each
isotype was subjected to a second round of subclass separation.

Production and Characterization of Monoclonal Anti-idiotypic Antibodies
(monoclonal GLXA-Ab$_2$)

Monoclonal Ab$_1$ (89MS30) IgG was isolated from a protein A chromato-
graphy column as set forth above. The keyhole limpet hemocyanin conjugation
was carried out by using glutaraldehyde. Briefly, 1.5 mg of IgG was mixed with
0.05 mg KLH, (Sigma Chemical Co., MO) (approximately 1 molar of IgG per 50
amino acid of KLH) in equal volume of 0.2 % of glutaraldehyde in PBS, in-
cubated at room temperature with gentle stirring. One hour later, I M of glycine
was added to make a final concentration of 0.02M and incubated for another

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hour at room temperature. The conjugate was then dialyzed against PBS overnight.

Four days after the last boost (see immunization), spleen cells were isolated from two mice which had the highest titer. Fusion was made with mouse myeloma cell line Sp 2/0-Ag14 according to the technigues initially develped by Kohler and Milstein (Nature, 1975) and modified (Goldsby, A practical Guide to Making Hybridomas in Nucleic Acid & Monoclonal Antibody Probes, Swaminathn & Prokask, Eds. Deller. N.Y. pg. 367, 1989). Feeder cell layers were from spleens of mice 8 weeks old.

Anti-idiotypic antisera from immunized mice and supernatants from cloned cells were detected by a sandwich ELISA (Uytdehaag et al, J. Immunol. 134: 1225, 1985, Hiroshima et al, J. Immunol. 144: 224, 1990). Briefly, polystyrene Immulon II microtiter plates (Dynatech Laboratories, Inc., VA) were coated with 100 ul of 1 ug monoclonal Ab_1 IgG in 0.1 M carbonate buffer, pH 8.9 overnight at 4° C. The unbound IgG was removed and the wells were blocked by 3% BSA/PBS for 1 hour at 37° C. After washing, serial diluted antisera or 100 ul of culture supernatant from wells with hybridoma cells was added. After 1 hour incubation at 37° C and washing, 1 ug of biotin conjugated monoclonal Ab_1 IgG was added to each well and the reactivity was detected by the addition of streptavidin-horse-radish peroxidase (Jackson Immune Research Labs., PA). One hour later, TMB peroxidase substrate (Kirferggaard & Perry laboratories, Inc., MD) was added. The absorbance was read at 405 nm in a microplate reader. As positive and negative controls, immune sera taken before the fusion (1:10 dilution) and medium alone were used.

The inhibition of the binding of monclonal $GLXA-Ab_1$ to GLXA by mouse antiserum and the supernatant of the clones was determined by immune-chemi-luminmetric assay as described above.

Ten BALB/cByJ mice were injected with pristane (2,6,10,14-tetra-methylpentadecane, Sigma Chemical Co., MO), 1 ml per mouse intraperitoneally.

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Ten days later, the mice were injected with approximately 2×10^6 monoclonal GLXA-Ab$_2$ (91 MS441) producing hybridoma cells. The ascites was harvested by using a Vacutainer 20 G blood collection needle (Becton Dickinson, NJ.) about ten days later. The ascites was clarified by centrifugation, 2000 rpm for 10 minutes and stored at -20° C until use.

The isotyping was carried out by ELISA. The supernatant from clone 91 MS441, (100 ul), was coated in each well and incubated at 4° C overnight. After rinsing and blocking with 3% BSA/PBS for 2 hours, 100 ul of rabbit antiserum specific to mouse subclass: IgGl, IgG2a, IgG2b, IgG3, IgM, IgA, K chain or λ chain (Bio-Rad Laboratories, CAA) was added to each well in duplicate. The plate was incubated at room temperature for one hour. The rabbit antiserum was detected by horseradish peroxidase conjugated goat anti-rabbit (H and L) and TMB substrate (Kirkergaard & Perry Laboratories, Inc. MD).

The monoclonal GLXA-Ab$_2$ IgG was purified by affinity chromatography on a protein-G-sepharose column. Protein G-Sepharose 4B (Zymed, CA) (5 ml) was packed in a 0.5 x 9 cm column. The ascites (2 ml) was diluted 1:1 with 0.02M phosphate buffer, pH 7.3 and loaded on the column and washed with the same buffer until no protein was detected. The bound IgG was eluted with 0.1 M citric-glycine buffer, pH 2.6. The eluent was collected in 1 ml fraction which contained 50 ul of 1 M tris-saline buffer, pH 8.0. Fractions having UV absorption above 0.1 were pooled and dialyzed in PBS overnight at 4° C. The purified IgG was identified by SDS-PAGE for confirmation.

Polyclonal antibodies from a human patient diagnosed with a chlamydial infection and chlamydial EBs injected rabbits were tested for the recognition of monoclonal GLXA-Ab$_2$ by ELISA. The procedure was essentially the same as described above with the following exceptions. Briefly, the ELISA plate was coated with 1 ug of monclonal GLXA-Ab$_2$ per well in 0.075M PBS without coating buffer overnight at 4° C. The wells were rinsed with 0.05% Tween 20 in 0.075M PBS and blocked with 3% BSA/PBS for 2 hours at room temperature. Serial

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dilution of the patient's serum (92MS273), control human serum (88MS356), rabbit antisera (88MS188) or control rabbit serum (92MS450), 100 ul, was added to each well in duplicate. After 1 hour incubation at room temperature, the wells were washed 3 times and 100 ul of peroxidase conjugated goat anti-human or goat anti-rabbit IgG (Jackson ImmunoResearch Labs, MD) was added. TMB substrate was added after one hour incubation. The plate was read on a Vmax microplate reader (Molecular Devices Corp., CA).

Immune-dot blot assay was done by the same method as described above. The exception is that a PVDF sheet was coated with purified GLXA (100 ul) per lane in 0.075M PBS. Antisera taken from the mice which were immunized with monoclonal GLXA-Ab$_2$ IgG or normal mouse IgG were serially diluted with 3% BSA/PBS. The second antibody was horseradish peroxidase conjugated rabbit anti-mouse IgG (H and L). The photograph was taken by Kodak TMAX 100. The staining intensity of dot blot was scanned by a densitometer.

Production of Antigen

C. trachomatis serovar B (Har. 36) elementary bodies were propagated in a McCoy cell monolayer in a 2 liter roller bottle (Corning, PA) in HMEM with added L-glutamine (final concentration 0.5 mM), NaHCO$_3$ (final concentration 0.4 mM) and 10 % of fetal bovine sera (FBS). Briefly, the medium was removed from the bottle after confluence of the monolayer. Then, 0.5-3.0 ml of EBs suspension (depending on the density of the organisms) in 40 ml of cycloheximide overlay medium (COM) (Whittacker, MD) with added L-glutamine and FBS was inoculated and the bottle was rolled at 3 rpm at 35° C. Two hours later, 200 ml of COM was added and rolling continued for 48 to 72 hours. The supernatant was obtained after centrifugation to remove organisms and any cell debris.

GLXA was purified by two steps from the supernatant. First, the supernatant was passed though an octyl-sepharose CL4B column and eluted with 95% ethanol (Stuart and MacDonald, Current Microbiology, 11:123, 1984). The

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antigen in ethanol (antigen content equivalent to 50 ml heavily infected tissue culture supernatant) was concentrated to approximately 50 ul and resuspended in 0.1M phosphate buffer, pH 7.5. to I ml. Second, antigen thus prepared was further purified by affinity chromatography. The affinity column was prepared by conjugating monoclonal GLXA-Ab$_1$ IgG to Affi-Gel 10 (Bio-Rad Laboratories, CA) as instructed by the manufacturer. The sample was centrifuged and loaded on to the affinity column and incubated for 30 minutes at 4° C. The column was rinsed with 0.1 M phosphate buffer until the eluant became clear. GLXA was eluted in 5M of potassium thiocyanate (KSCN, Mallinckrodt Inc., KY) with about 15-20 ml and dialyzed against 0.075M PBS overnight.

GLXA used in the immune-dot blot assay of binding of GLXA and monoclonal GLXA-Ab$_2$ by GLXA-Ab$_3$ described below was isolated by a size exclusion method. Sepharose CL-CB column (2x100 cm) was equilibrated with 0.075M PBS. The supernatant from C. trachomatis serovar F cell culture was first centrifuged 5000 rpm to remove cell debris and approximately 20 ml was loaded onto the column. The flow rate was 0.5 ml/minute. GLXA in the eluant was detected with Magic Lite Analyzer (Ciba Corning Diagnostic Corp., MA). The protocol is as described as below.

Inhibition of the binding of monoclonal GLXA-Ab$_1$ IgG to GLXA by guinea pig GLXA-Ab$_2$ or rabbit GLXA-Ab$_3$ (antisera, IgG, IgG isotypes) was detected by chemiluminometric immunoassay with a Magic Lite Analyzer (Ciba Corning Diagnostic Corp., MA). The protocol for the inhibition was as follows: Affinity purified GLXA was diluted 1:5 with reagent A (Ciba Corning), mixed and aliquoted into plastic tubes (200 ul/tube, Sarstadt, NJ). A neutralizing solution Reagent B (Ciba Corning), (100) ul was added to each tube and mixed well. Serial dilutions of guinea pig GLXA-Ab$_2$ or rabbit GLXA-Ab$_3$ IgG, (50 ul) were added to each of the above tubes and incubated at room temperature. One hour

after the incubation, 100 ul acridium ester-labeled (AE-labeled) monoclonal GLXA-Ab₁ IgG in 1% BSA/PBS (between 220,000 to 240,000 relative light units, RLU) was added into the mixture and incubated for one hour. Then, polyclonal anti-chlamydial antibodies which were covalently bound to paramagnetic particles (500 ul) was added to each tube and incubated for another hour. The bound immune complex (paramagnetic particles-GLXA-GLXA-Ab ₁-AE labelled monoclonal GLXA-Ab₁ IgG) is separated from the unbound by subjecting the mixture to a magnetic field. The particles were rinsed 3 times. Bound AE-labeled antibody was measured by the Magic Lite Analyzer and RLU were recorded. The percentage of inhibition of binding was calculated as follows:

$$\% \text{ Inhibition} = \frac{\underline{RLU \text{ control } -RLU \text{ test}}}{RLU \text{ control}} \times 100$$

where control represents PBS and test represents either pre-immune or antisera (or IgG) added respectively.

A Bio-rad dot blot apparatus was used for a direct assay. Polyvinylidene fluoride (PVDF) membrane, 0.45 um, Immobilon-NC (Millipore, MA) was coated with the affinity-purified GLXA or chlamydial rLPS for 18 hours at 4° C. The membrane was then washed with 0.05 % Tween 20 in 0.075M PBS buffer, pH 7.2 and blocked for 2 hours at room temperature with 3 % BSA/PBS. After rinsing, serial dilutions of rabbit GLXA-Ab₃ IgG (9OMS699) (isolated by protein A affinity chromatography, method see above) or monoclonal Ab₁ IgG (89MS30) were added (100 ul per well), incubated at room temperature for 2 hours, and washed to remove unbound IgG. The PVDF sheet was removed from the apparatus and blocked with 3 % BSA/PBS for two hours with gentle agitation, then probed with 1:1000 dilution of peroxidase conjugated goat anti-rabbit IgG (H and L) or rabbit anti-mouse IgG (H and L) (Jackson ImmunoResearch Labs., PA) for 1 hour at room temperature. Unbound conjugated antibody was removed by washing in a petri dish for 4 times, 5 minutes with agitation. The binding was

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detected by 4 CN (4-chloro-l-naphthol) membrane peroxidase substrate system (Kirkegaard & Perry Laboratories Inc., MD). Assays were carried out in duplicate.

Monoclonal GLXA-Ab$_1$ or rabbit GLXA-Ab$_3$ IgGs were labeled with biotin. Briefly, the GLXA-IgG was first dialyzed against 0.1 M sodium borate, pH 8.8 for 4 hours at 4° C. Then, 200 ug of biotinamidocaproate N-hydroxysuccinimide ester (Sigma Chemical Co., MO) per mg of IgG was added, and incubated at room temperature for 4 hours. Finally, 20 ul of 1 M NH$_4$Cl per 250 ul of ester was used and incubated for 10 minutes at room temperature to stop the reaction. Labeled IgGs were dialyzed against 0.075M PBS for 3 days at 4° C.

Confluent McCoy cell monolayers were grown on cover slips in a 24 well plate (Falcon, NJ) for 24 hours. The medium was removed from each well before 200 ul of C. trachomatis serovar B elementary bodies (Har 36) or 200 ul of HMEM alone were applied to each well. The inclusion forming units (IFU) was approximately 1000/200 ul. Immediately after the application, 200 ul of cycloheximide overlay medium (COM) with L-glutamine and FBS was added to each well. The mixture was incubated at 37° C for two hours and the medium was replaced by 1 ml COM per well. The plates were then cultured for 48 hours at 37° C in a humidified incubator with 5% CO$_2$.

After the incubation, cell cover slips were washed with 0.075M PBS twice, 5 minutes each. The coverslips were then blotted with tissue carefully to remove buffer on the cover slips. Then 400 ul of 50 ug/ml biotin-labeled monoclonal GLXA-Ab$_1$ or 400 ul of 1.20 mg/ml of biotin-labeled GLXA-Ab$_3$ was added to the cover slips and incubated at 37° C for 1 hour. The cover slips were rinsed twice with PBS (five minutes each rinse). Four hundred ul of fluorescein-streptavidin 1:50 in 1% of BSA/PBS was added and incubated for 1 hour. The unbounded fluorescein-streptavidin was removed as above. Fluorescence was detected by photograph with an Olympus 25, 35mm camera (ASA 400 TMAX black and white

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Kodak film) mounted on a Zeiss A.7082 Oberkichen microscope, illuminated with a 12v/100z halogen lamp.

Neutralization of Chlamydia by GLXA-Ab₃ (90MS699) in vivo

The rabbit pre-immune and GLXA-Ab₃ IgG, normal mouse and monoclonal GLXA-Ab₁ IgG were filter sterilized. The IgGs (0.2 mg/ml) were mixed (1:1) respectively with C. trachomatis serovar C (TW-3) elementary bodies (200 inclusion-forming units/20ul). The mixtures and non-treated EBs (as a control) were incubated at 37° C for 45 minutes, with gentle shaking every 15 minutes.

After the incubation, approximately 2 ug IgG plus 1000 IFU in 20 ul was inoculated onto inferior fornix of each eye of eight monkeys, four with GLXA-Ab₃ IgG plus EBs, two with pre-immune IgG plus EBs and two with EBs only. At the same time, 100 ul of each type of mixture was used to infect 10 wells of 2-day-old McCoy cell monolayer coverslip in a 48 well tissue culture plate for determination of the infectivity of the inoculum. Culture methods are described below.

Conjunctival swabs were taken by sweeping the interior tarsus and fornix, the lateral fornix, the superior tarsus and fornix, and the medial fornix on the day prior to inoculation, and on days 2, 6, 9, 13, and 20 after the inoculation. The swabs were immediately immersed in 2 ml collection medium and disrupted by vortexing for 2 minutes in the collection medium.

Conjunctival swabs were first disrupted by vortexing for two minutes in the collection medium to collect EBs from the swabs. Then 200 ul of this collection medium was inoculated onto McCoy cell monolayer cover slips. The cells in a 48 well tissue culture plate were grown for two days, 5 wells for each sample. For an in vitro neutralization experiment, 100 ul of the mixture was inoculated

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onto the monolayer cover slips of 10 wells for each sample. The inoculated plates were then centrifuged at 1,000 x g for 1 hour at room temperature. The plates were incubated at 37° C for 2 hours and the medium was replaced by 1 ml of 10 % FBS in COM (Whittaker, MD). The plates were cultured at 37° C in a humidified incubator with 5% CO_2. for 48 hours. The culture medium was aspirated from each well after the incubation. The monolayer coverslips were washed once with 0.075M PBS, fixed with ethanol for 5 minutes and then rinsed with H_2O twice. The fluorescein-labeled rabbit anti-chlamydial monoclonal antibody with Evans blue counterstain (Syva, Co., CA.) (25 ul) per well was added and incubated for 30 minutes at 37 °C. After the incubation, the wells were rinsed with H_2O twice and a cover slip was mounted to each well with mounting fluid. The inclusion bodies were counted by inverting the plate on a fluorescence microscope.

The conjunctival swab (scraping) from each eye of infected primates was pressed on alcohol-precleaned glass slides. The slides were air dried and fixed with cold acetone for 5 minutes. Slides were then dried and 30 ul of fluorescein-labeled monoclonal antibody reagent with Evans blue counterstain (MicroTrak Chlamydia Direct Reagent, Syva Co. CA) were overlaid. The incubation was carried out in a covered, moist chamber. After 15 minutes, slides were turned on edge to remove excess stain and rinsed with deionized water for 10 seconds. Slides were allowed to completely air dry. A cover slip was placed on each slide with mounting medium (Syva Co., CA) and sealed with fingernail polish. Slides were examined under a fluorescence microscope at 500x and 1250x. The infectious titer was scored on a semi-quantitative scale 0-4+: 0 is negative, 0.5 is negative on first passage and positive to any degree of second passage, 1 is for 1 to 9 inclusions per well on first passage and so on.

The clinical response of each eye was graded as ten clinical signs (Taylor et al, Invest. Opthalmol. Vis. Sci. 29:847, 1988): the follicular response in the lumbar, limbal, superior tarsal, superior fornix, and inferior fornix portion of the conjunctiva hyperemia or injection of the bulbar, superior tarsal, superior fornix, and inferior fornix conjunctiva and ocular discharge. The clinical response was graded on a scale of 0 to 3 for each of 10 signs of conjunctival inflammation to obtain a total inflammatory score for each monkey. The examiner was unaware about the allocation of the monkeys. The means of the scores were used to describe the response of a group of monkeys.

Total RNA was prepared from conjunctival swab samples taken from GLXA-Ab$_3$ or normal rabbit IgG treated monkeys on the day before the challenge and on day 2, 6, 9, 12, and 20 after the challenge. The swab samples were first homogenized in phenol at 65% in the buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA) and 1 % SDS. RNA was precipitated and redissolved in the same buffer. This preparation of RNA was extracted several times with phenol:chloroform (3:1), resuspended and treated extensively with DNase I. The quality of RNA preparation was monitored by ethidium bromide-UV visualization after separation on formaldehyde-agarose gels.

The probe used was a DNA fragment containing the 16S and 23S ribosomal RNA and flanking sequences which was excised from the chlamydial genomic plasmid clone pL2, 434Scl-IA (Cheema et al, The Amer. J. Med. Sci. 302:261-268, 1991). The DNA fragment was labelled by nick translation using ^{32}PdCTP, 800 Ci/m mole (Amersham Corp., IL). The specific activity for all restriction fragment probes was about 106 cpm/ug DNA. Slots on each blot included 1 ug of monkey or human-derived total RNA, 10 pg pure C. trachomatis or C serovar RNA (positive control), 3 ug yeast or rat RNA, buffer alone and 1 ug RNA from swabs of each monkey taken prior to infection (negative control). RNA

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was fixed to 0.22 um filter (Schleicher and Schuell Corp., NH). The hybridization results were visualized via autoradiography at -70° C using X-OMAT AR film (Kodak, New York).

Inoculation and specimens

C. trachomatis serovar C (TW-3) elementary bodies 5000/20ul were inoculated onto each eye of the mice which were immunized with monoclonal Ab_2 or normal mouse IgG. On the day before the inoculation and on day 7, 10, 14, 21, 28 and 35 after the inoculation, conjunctiva were swabbed from each eye. The area included the inferior tarsus and fornix, the lateral fornix, the superior tarsus and fornix, and the medial fornix. The conjunctival swabs were immediately immersed in the collection medium and disrupted for two minutes by vortex and kept on ice until culturing.

Detection and Characterization of Polyclonal Anti-idiotypic Antibodies

The immunogen which was used to produce the anti-idiotypic antibodies in guinea pigs was a monoclonal antibody identified as 89MS30 (monoclonal GLXA-Ab_1). It was originally produced by immunization of BALB/cByJ mice with chlamydial elementary bodies propagated in embryotic egg. Mice spleenocytes were fused with Spa/0-Agl4 myeloma cells and the clone was screened. The clone (89MS30) reacted to all 15 serovars of C.trachomatis, C. pneumoniae, and C. psittaci 6BC and mouse meningopneumonitis by EIA, demonstrating recognition of a genus specific antigen. The IgG was isotyped as IgG2b by ELISA using rabbit anti-mouse antiserum (Bio-Rad Laboratories, CA). The monoclonal GLXA-Ab IgG was isolated from the ascites with rec-protein A sepharose 4B conjugate column (ZYMED, CA). Inbred guinea pigs (Hartley 13) were immunized and boosted with monoclonal GLXA-Ab_1 IgG, 150 ug each in the presence of Maalox, as an adjuvant. Pre-immune sera and antisera were obtained by heart puncture and centrifugation. Five immunized guinea pigs demonstrated strong immune

responses against monoclonal Ab₁ IgG by ELISA. It was demonstrated that the
anti-monoclonal GLXA-Ab₁ IgG titer was more than 1 to 20,000 one week after
the first boost. These guinea pig antisera kept increasing two weeks after the
boost as shown in Figure 1.

In order to remove guinea pig anti-mouse antibodies that are directed
against epitopes other than the idiotopes present on the hypervariable regions of
the monoclonal Ab₁ IgG molecules, the guinea pig antisera was absorbed with
normal mouse IgG. The absorption was carried out by affinity chromatography.
The column was made by conjugating normal mouse IgG to Affi-Gel 10. The
conjugation was evaluated by detecting the amount of unbound protein at
absorbance at 280 nm. The highest optical density from a fraction was 0.23, con-
taining 0.18 mg of protein by the Bradford Assay (Bio-Rad Laboratories, CA).
The total mouse IgG used in the conjugation was 50 mg, demonstrating that the
conjugation was successful. The guinea pig antisera were loaded onto the
column and eluted with 0.075M PBS, pH 7.2. This procedure was repeated using
a newly prepared column. The antisera before and after the absorption was
tested and compared with pre-immune sera for the reactivity to normal mouse
IgG by ELISA. The concentration of each antiserum for the assay was normal-
ized. The result showed that sera after the absorption had the same reactivity
against normal mouse IgG as pre-immune sera (Fig. 2). The unabsorbed antisera
had 5 times more reactivity, indicating that all antibodies specific to epitopes
other than idiotypes have been completely removed.

If guinea pig antisera contain the specific anti-idiotypic antibody against
monoclonal GLXA-Ab₁ IgG molecules, the direct effect is that it would inhibit the
binding of antigen, GLXA, to the monoclonal antibody, monoclonal GLXA-Ab₁. In
other words, the antisera bind to the complementarity-determining region of
monoclonal GLXA-Ab₁ IgG, thus preventing GLXA from binding to the active site.
To test this, competition of binding was performed by chemiluminometric im-

munoassay. Serial dilutions of guinea pig pre-immune sera, unabsorbed antisera, or absorbed antisera were incubated with GLXA which was isolated by immune-affinity chromatography. After one hour at room temperature, the mixtures were incubated with monoclonal GLXA-Ab₁ IgG conjugated with acridium ester for an additional hour. Solid phase paramagnetic particles were added and a RLU was determined. As shown in Figure 3, when the guinea pig antisera was diluted 1:40, 95% of the binding of monoclonal Ab₁ to GLXA was inhibited by both unabsorbed and absorbed antisera, compared with 15·% for pre-immune serum. In addition, the unabsorbed antisera had nearly identical inhibition as absorbed antisera at equivalent protein concentration, indicating that no anti-idiotypic antibodies were removed by absorption. Therefore, a competitive anti-idiotypic antibody had been generated in guinea pigs.

Previous experiments had shown that different isotypes of guinea pig anti-idiotype exert different effects on the idiotype production. IgG-1 isotype enhances the idiotype production, whereas IgG-2 isotype inhibits the idiotype clone. To test the inhibition of different isotype of guinea pig anti-idiotypic IgG, subclasses of IgG were isolated from absorbed guinea pig anti-idiotypic antisera. A step pH gradient of phosphate-citrate buffer was used. IgG-1 and IgG-2 were separated and isolated on a protein A affinity column. The elution profile of the isotypes, IgG-1 and IgG-2 is shown in Figure 4. Each peak was identified by immuno-electrophoresis (IEP). Goat anti-guinea pig IgG (Bethyl, TX) was used to precipitate the bands. The first peak which is composed of IgG-1 formed a single band by goat anti-guinea pig antisera. The first peak was then repurified by the same method.

The competition by different subclasses of IgG was carried out again by chemiluminometric immunoassay using guinea pig anti-idiotypic antibodies of either IgG-1 or IgG-2 isotypes. Figure 5 shows that 0.4 ug IgG-I was able to inhibit the binding of monoclonal GLXA-Ab₁ to GLXA by 50 %. The total inhibition

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occurred when 100 ug was used. This was essentially the same concentration needed when unlabeled monoclonal GLXA-Ab$_1$ IgG was used. IgG-2 showed little, if any, inhibition, approximately 25 % of the binding of monoclonal GLXA-Ab$_1$ to GLXA with 1.0 ug. These data demonstrate that the IgG-1 subclass of anti-idiotypic IgG was at least 5 times more inhibitory than the IgG-2 subclass.

The evidence obtained showed that guinea pig anti-idiotypic IgG-I was against the hypervariable region of monoclonal GLXA-Ab$_1$ IgG and also inhibited its binding to GLXA. The final criteria of an internal image of the anti-idiotypic antibodies is to confirm structurally that the Ab$_2$ is Ab$_2$ β , not Ab$_2$ α. Since Ab$_2$ α binds to the framework portion of immunoglobulins, it can also inhibit the binding of Ab$_1$ to the cognate antigen. To confirm that guinea pig anti-idiotypic IgG-I is Ab$_2$, the isotype IgG-1 was used to produce an anti-anti-idiotypic antibody (GLXA-Ab$_3$) which can recognize the GLXA epitope in an animal which has never been exposed to GLXA antigen, (Ertl et al,Proc. Natl. Acad. Sci. USA 81:2850, 1988). Three New Zealand white rabbits were immunized with guinea pig anti-idiotypic IgG-I in the presence of adjuvant, Maalox (alum). The antisera from one rabbit (S2) were tested against IgG-I by ELISA (Fig. 6). The titer increased with time after the immunization. The titer was much higher than 20,000 compared with pre-immune sera three weeks after the second immunization.

The dot blot apparatus (Bio-Rad Laboratories, CA) was used to detect the reactivity of rabbit antisera to GLXA. Antisera from two rabbits were tested. Since monoclonal GLXA-Ab$_1$ is cross-reactive to chlamydial LPS, polyvinylidene fluoride (PVDF) membrane was coated with both GLXA and chlamydial rLPS. Both rabbits antisera recognized GLXA and LPS with high reactivity. When IgG from rabbit (S2) (90MS699) was used, the dots were positive at a concentration of 0.006 ug per lane. Pre-immune IgG did not react. The IgG isolated from this antisera was tested for its capacity of inhibiting the binding of monoclonal GLXA-

Ab$_1$ to GLXA, since GLXA-Ab$_3$ produced by the internal image, GLXA-Ab$_2$ should exhibit a similar binding. This is confirmed as shown in Figure 7. The inhibition by GLXA-Ab$_3$ increases with concentration, but about five times less inhibitory compared to unlabeled monoclonal Ab$_1$. This demonstrates that the guinea pig anti-idiotypic antibody IgG-I is the internal image of antigen, GLXA. GLXA-Ab$_3$ IgG was further tested for the recognition of elementary bodies of C. trachomatis in vitro.

Monoclonal GLXA-Ab$_1$ and GLXA-Ab$_3$ IgG were conjugated with biotin by glutaldehyde. Labeled monoclonal GLXA-Ab$_1$ or GLXA-Ab$_3$ was incubated with McCoy cell monolayer on coverslips which were infected with C. trachomatis serovar B (Har 36) elementary bodies for 48 hours. Non-infected monolayers were used as control. The florescence staining pattern of the elementary bodies by monoclonal GLXA-Ab$_1$ and polyclonal GLXA-Ab$_3$ IgGs demonstrated that GLXA-Ab$_3$ not only recognized the purified form, but also the native form of GLXA.

Neutralization of the Chlamydial Infection in Primates by GLXA-Ab$_3$ IgG
    (9OMS699)

The next question concerned the ability of monoclonal GLXA-Ab$_1$ or GLXA-Ab$_3$ to neutralize chlamydia elementary bodies and thus protect host cells from infection. The experimental approaches utilized to answer this question involved neutralization of infection in cultured cells and neutralization of the infection in primate conjunctiva.

Neutralization in primate conjunctivae was determined by preincubating organisms with antibody IgG and detection of the effect on ocular infection. The IgG fraction of monoclonal GLXA-Ab$_1$ or Ab$_3$ was incubated with elementary

bodies of C. trachomatis serovar C. Normal mouse or rabbit pre-immune IgG as
well as no immunoglobulin added served as controls. The mixture was inoculated
to each eye of the primate. On the day before the and after the inoculation, con-
junctival swabs were taken by sweeping different areas of the conjunctivae (see
above). Ocular chlamydial infection in primates was determined by cell culture
assay which included second-passage on days post-infection. As shown in Table
1, three primates were infected with monoclonal GLXA-Ab₁ treated EBs on the
left eyes, GLXA-Ab₃ treated EBs on the right. At the same time, two primates
were infected with normal mouse IgG treated EBs (shown as NI) or EBs alone
on the left and right eyes alternatively. The same method applied to pre-immune
rabbit IgG (shown as N3). All eyes inoculated with monoclonal GLXA-Ab₁ treated
EBs were positive at least once (primate No. 515, 84, and 26). However, only
one eye of the GLXA- Ab₃ treated eye was positive once, at day 10 post-infec-
tion, two of them were never positive (primate No. 515, 84 and 26). Eyes
inoculated with normal mouse or pre-immune rabbit IgG treated EBs were all
positive at least once (primate No. 563, 20, 17 and 329). The untreated EBs
(shown as C) produced infection in two of the four eyes involved (primate No.
563, 20, 17 and 329). Although the data points are diminutive, they do show that
monoclonal GLXA-Ab₁ does not neutralize chlamydia in primate conjunctiva. On
the other hand, it suggests that GLXA-Ab₃ is neutralizing. In order to confirm that
GLXA-Ab₃ does neutralize, a number of tests were carried out, including: (A,)
neutralization in cell culture (B), neutralization in primate conjunctiva (C), detec-
tion by clinical culture assay (D), detection by direct fluorescence antibody
cytology (E), chlamydia specific RNA probe hybridization and (F) determination of
the severity of ocular infection by clinical scoring.

Table 1.

Neutralization of chlamydial infection in primate conjunctivae
by Ab$_3$ but not mAb$_1$

Primate	Eye	Ab[a]	Day Following Infection				
			0	3	7	10	14
515	L	mAb$_1$	-	-	+	+	-
	R	Ab$_3$	-	-	-	-	-
563	L	C	-	-	·-	-	-
	R	N$_1$	-	-	-	+	-
84	L	mAb$_1$	-	-	-	+	+
	R	Ab$_3$	-	-	-	+	-
20	L	N$_3$	-	-	-	+	-
	R	C	-	-	-	-	-
26	L	mAb$_1$	-	-	+	-	-
	R	Ab$_3$	-	-	-	-	-
17	L	N$_1$	-	-	-	+	+
	R	C	-	-	-	+	+
329	L	C	-	-	+	-	-
	R	N3	-	-	-	+	+

[a]EBs treated with normal mouse IgG(N1), pre-immune rabbit IgG (N3) or EBs
alone (C) were controls.

GLXA-Ab$_3$ neutralizes the chlamydial infection in vitro.

In vitro, cell culture assay was carried out with GLXA-Ab$_3$ and pre-immune
rabbit antibody. The pre-immune rabbit and GLXA-Ab$_3$ (9OMS699) IgGs were
isolated by protein A affinity chromatography. The mixture of 10 ug of each IgG
and 100 ul serovar C EBs (1000 IFU/ml) or EBs alone were inoculated onto wells
containing McCoy cell monolayers. Ten wells per sample were used. Inclusion

bodies were detected by FITC-conjugated monoclonal anti-chlamydia antibody 48 hours after the incubation. IFU/ml was based on 15 fields per well. As is shown in Table 2, Ab_3 IgG reduced the infectivity 3 times higher compared to pre-immune IgG, 5 times higher compared to EBs alone, indicating neutralization by $GLXA-Ab_3$.

Table 2.

In vitro neutralization of chlamydial infection by Ab_3 IgG

EBs treated with	Mean IFU/15 Fields + S.E.M.
Ab_3	34.1 + 7.2
Normal IgG	93.8 + 22.4
None	155.3 + 5.5

$GLXA-Ab_3$ neutralizes the chlamydial infection in primates

Eight primates were randomly divided into three groups in this experiment. In the eyes, four primates received with purified EBs previously incubated with $GLXA-Ab_3$ IgG (eight eyes), two received EBs previously incubated with pre-immune IgG (four eyes) and two received untreated EBs (four eyes). On the examining day, the con-junctival swabs were taken and cell cultured. Since there is no significant differ-ences between the recipients of pre-immune IgG and EBs alone, the results are presented as 8 experimental eyes and 8 control eyes. Cell culture results are expressed as IFU/ml based on counting inclusions in 15 fields for two wells per sample. As shown in Table 3, 20 days after the challenge, 1 of eight eyes was positive compared to eight out of eight eyes that were positive in the control group. When the accumulated results were examined, with $GLXA-Ab_3$, 9 of 40 (22.5%) were positive, in the contrast, 36 of 40 (90%) were positive without $GLXA-Ab_3$.

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Table 3.

Neutralization of chlamydial infection in primate conjunctivae by

Ab$_3$ IgG by cell culture assay[a]

| Day of Experiment IgG Control | Ab$_3$ | No. of Eye Culture Positive | | |
		Preimmune	None[b]	Combined
0	0/8	0/4	0/4	0/8
2	3/8	2/4	3/4	5/8
6	3/8	4/4	4/4	8/8
9	1/8	3/4	4/4	7/8
12	1/8	4/4	4/4	8/8
20	1/8	4/4	4/4	8/8
	9/40	17/20	19/20	36/40

a. Only one first passage negative sample was second-passage positive.
b. EBs were previously incubated without antibody.

In an parallel assay, direct fluorescence antibody cytology assay (DFA) was also carried out to evaluate the numbers of EBs from the conjunctival swabs. The conjunctival swab samples were fixed onto slides and stained with FITC-labeled monoclonal antibody against C. trachomatis. It was considered to be positive when 5 or more characteristic elementary bodies were seen on each slide (Micro Trak, Syva Co. CA). As shown in Table 4, EBs were detected in the GLXA-Ab$_3$ treated group on only two occasions, day 6 and 12 post-infection, while the remainder were positive through day 20. Only one eye was EB negative in the non-treated group at day 2, that was probably an artifact. EBs were detected in all eyes in this group for the remainder of the experiment. On the last examination day (day 20), none of the eyes treated with GLXA-Ab$_3$ was positive, while 8 of 8 were positive in the combined control group. In addition, DFA and culture results are completely

congruent.

Table 4.

Neutralization of chlamydial infection by Ab_3 using direct
fluorescence antibody cytometry assay (DFA)[a]

| Day of Experiment | Ab_3 | No. of Eye Culture Positive | | | |
		Preimmune IgG	None[b]	Combined	Control
0		0/8	0/4	0/4	0/8
2		0/8	2/4	0/4	5/8
6		1/8	2/4	4/4	6/8
9		0/8	4/4	4/4	8/8
12		1/8	4/4	4/4	8/8
20		0/8	4/4	4/4	8/8
		2/40	16/20	19/20	35/40

[a]DFA was considered positive if 4 EBs were found on a slide.

[b]EBs were considered previously incubated without antibody

GLXA-Ab_3 substantially attenuates the chlamydial replication in conjunctival
infection

Chlamydial specific ribosomal RNA had been examined from those primate
conjunctival swabs by a DNA probe (Cheema et al,The Ameri. J. Med. Sci.
302:261-268, 1991). Total RNA was extracted from conjunctival swabs taken from
primate eyes. RNA from serovar C EBs, human or yeast were used as control. [32]P-
chlamydial DNA encoding ribosomal RNA16S and 23S genes was used to detect
chlamydial specific RNA in a Northern slot-blot hybridization assay. As shown in
Figure 8, control eyes (infected either with pre-immune rabbit IgG plus EBs or EBs
alone) uniformly show significant levels of chlamydial RNA at all time points
examined, similar RNA samples prepared from the eyes of GLXA-Ab_3-treated

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organism show significantly attenuated levels of chlamydial RNA. This indicates that the neutralization happens at the very early stage of the infection.

The degree of conjunctival inflammation after inoculation with EBs previously incubated with either GLXA-Ab$_3$ or pre-immune IgG or without previous incubation was evaluated by clinical response. The clinical response was graded based on a total clinical disease scores (TCDS) derived from 10 clinical features of inflammation (Taylor et al, Invest. Opthalmol. Vis. Sci. 29: 1847, 1988). The accumulative disease scores were obtained for each group of primates by examining 10 signs existing in the conjunctiva. As shown in Figure 9, recipients of GLXA-Ab$_3$ developed very little clinical disease and this declined after day 8. Control animals continued to develop severe disease through day 21 post-challenge. This pathological finding is consistent with cell culture, DFA and RNA hybridization data.

Generation of And Characterization of Hybridoma Cell Lines Producing Anti-idiotypic Antibody.

Five syngeneic mice (BALB/cByJ) were immunized intraperitoneally with KLH conjugated monoclonal GLXA-Ab$_1$ IgG in the presence of Freund's complete adjuvant. The anti-idiotypic anti-sera against monoclonal GLXA-Ab$_1$ IgG from these mice were detected by sandwich ELISA. The titer was about 1:1000 three weeks after the immunization (data not shown). Fusions were made between Sp2/0-Agl4 cells and spleen cells from two mice which had the highest titer against monoclonal GLXA-Ab$_1$. The size of the spleens were almost twice the size of normal spleen. The clones were screened by the same method for mouse antisera testing. The pool of mice antisera or pre-immune sera diluted 1:10 was used as controls in all screening tests. Among 283 wells screened, 8 were considered positive against GLXA-mAb$_1$ with a titer two times or higher than negative control. These hybridomas were cloned. One clone (91 MS441) had the highest titer in ELISA and was propagated. The ascites was produced and IgG

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isolated. The isotype of IgG of this clone was identified to be IgGl, having a K light chain (Bio-Rad Laboratories, CA). The fusion results was summarized in Table 5.

Table 5.

Summary of fusion for anti-idiotypic antibodies

Immunized BALB/cByJ Mice	Anti-id Titer in Supernatant	Total Wells with Growing Hybridoma	Wells Positive of Anti-id (%)	Stable Cloned Anti-id Lines (% of Total)
2	1:1000	283	8 (2.8)	1 (0.35)
	Light chain:K		Heavy chain:IgG'	

The supernatant from positive clones were further tested for the inhibition of the binding of monoclonal GLXA-Ab$_1$ to GLXA by chemiluminometric immunoassay. Total inhibition of the binding was found from the supernatants of 4 clones without dilution. Two weeks later only one clone (91 MS441) was found stable. As shown in Figure 10, at a dilution of 1:10, the supernatant from this clone inhibits the binding of monoclonal GLXA-Ab$_1$ to GLXA by 80 % compared to 0% of a non-positive clone (91 MS442) from the same fusion. This demonstrates that an anti-idiotypic clone was produced. It is identified as monoclonal GLXA-Ab$_2$.

As an internal image of an antigenic determinant, the anti-idiotypic antibody should be recognized by any specific antisera which is against this epitope regardless of species. In order to test this property of the IgG produce by clone (91 MS441), human serum from a patient clinically diagnosed as chlamydia positive was used. Since the isotype of monoclonal GLXA-Ab$_2$ is IgGl, the purification of the IgG was carried out by protein G affinity chromatography. The isolated monoclonal GLXA-Ab$_2$ IgG was coated on a microtiter plate and tested for reactivity with the patient's serum (91 MS253) by ELISA. Human serum without clinically diagnosed chlamydial infection (88MS356) was used as a negative control. The results showed that the patient's serum had a titer which was more than 1:2000 against monoclonal GLXA-Ab$_2$ (Fig. 11). This demonstrates that infected human antisera

has the specific antibody against monoclonal GLXA-Ab$_2$, implying that monoclonal GLXA-Ab$_2$ is the internal image of GLXA.

The specific recognition of monoclonal GLXA-Ab$_2$ by a rabbit anti-chlamydial antisera (88MS188) was also tested in the similar way. The rabbit antiserum was produced by inoculating EBs. As shown in Figure 12, rabbit antisera has higher binding to monoclonal GLXA-Ab$_2$ than pre-immune serum, but lower than expected. The difference between the binding is seen to the dilution factor of 1:1000. This shows that rabbit antiserum which is against chlamydia has the specific antibody recognizing monoclonal GLXA-Ab$_2$, an additional evidence for monoclonal GLXA-Ab$_2$ as an internal image.

The anti-idiotypic antibody as an internal image can raise antigen specific antisera in animals that have never been exposed to that antigen. in this case, if monoclonal GLXA-Ab$_2$ (91 MS441) is the internal image of GLXA, (that is bearing the same antigenic structure), it should produce an anti-GLXA antibody in the same strain BALB/c ByJ mice in spite of the fact that they have never been exposed to GLXA. In an initial experiment, 8 mice were immunized with monoclonal Ab$_2$ IgG, four mice with normal mouse IgG. No adjuvant was used in the subcutaneous injection. Seven days and 14 days after the immunization and one boost, antisera were obtained from these mice by sacrificing half of the mice in each group. Immune-dot blot assay was carried out with purified GLXA. The dot blot showed that mice immunized with monoclonal GLXA-Ab$_2$ IgG had a titer as high as 1:800 compared to 1:100 or less of the mice immunized with normal mouse IgG. The blots were further quantified by a densitometer. When diluted to 1:100 after the first boost, the antisera from all four mice bind to GLXA whereas no binding was seen in the control group even when diluted 1:50. This demonstrates that this antisera is solely elicited by the paratope of the anti-idiotype because the recipients are syngeneic.

GLXA-Ab$_3$ antisera which specifically bind to GLXA were also tested for the inhibition of the binding of monoclonal GLXA-Ab$_1$ by chemiluminometric immunoassay. At a dilution of 1:25, GLXA-Ab$_3$ inhibits 90% of the binding compared to 18 % of inhibition by the control antisera day 8 post immunization. This is equal to the same inhibition demonstrated by 10 ug of unlabeled monoclonal GLXA-Ab$_1$. The same inhibition was found in the antisera on day 14, one week after the first boost with the same amount (50 ug/mouse) of IgG. It is noted that inhibition percentage dropped from 70% on day 7 to about 50 % on day 14 when diluted 1:100.

Immunization with Monoclonal GLXA-Ab$_2$ Protects Mice from Chlamydial Infection

BALB/c ByJ mice were used as an ocular chlamydial infection animal model in this study. The first experiment was carried out with 39 mice. Twenty mice were immunized subcutaneously with 50 ug of monoclonal GLXA-Ab$_2$ and nineteen with normal mouse IgG without any adjuvant. They were given a total of three injections, one week apart. One week after the last injection, the mice were inoculated with C. trachomatis serovar C elementary bodies (5000 IFU in each eye). The conjunctival swabs were taken from each eye of the mice on day 0, 7, 14, and 21 post infection. Samples from conjunctival swabs were collected and cultured in McCoy cell monolayers for 48 hours. Inclusion bodies were counted, and 5 inclusions were considered as positive in 15 fields. As shown in Figure 13, mice immunized with monoclonal GLXA-Ab$_2$ had half the infectivity compared with mice immunized with normal mouse IgG. In a second experiment (twenty mice in each group), the immunization was repeated in the similar manner. However, they received 10^6 IFU per eye, 100 times the number of EBs as used in the first experiment. Surprisingly, almost the same protection curve found in the mice (Fig. 14), suggesting that monoclonal GLXA-Ab$_2$ elicits a vigorous host defense response.

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In a third experiment, aluminum hydroxide (alum) was used in the immunization. Ten mice were immunized with monoclonal GLXA-Ab$_2$ in the presence of adjuvant, eight mice immunized without adjuvant and sixteen mice immunized with normal mouse IgG in the presence of Maalox. The immunization procedure is the same as the previous ones. When Maalox was used in the immunization, the titer of the anti-anti-idiotypic antisera in mice was doubled as tested against GLXA in immune-dot blot. The higher titered antisera in the adjuvant treated mice demonstrate more effective protection in the mice from the ocular infection compared to the ones immunized without alum (Fig. 15). However, mice immunized with monoclonal GLXA-Ab$_2$ had significantly less infection than those receiving normal mouse IgG plus alum. In addition, the infection was cleared by post challenge day 28. In the fourth experiment, eight mice were immunized with monoclonal GLXA-Ab$_2$ and eight with normal mouse IgG in the presence of alum. The immunization and inoculation protocols are the same as before. However, as shown in Figure 16 (A), on day 7, both groups had the same degree of infection, 50 % of the eyes are infected. However, beginning on day 10, a significant difference of the infection is seen between the two groups. For the group immunized with monoclonal GLXA-Ab$_2$ IgG, on day 14, two out of eight eyes were positive, on day 22, one out of eight. No eye was positive on day 28, showing a clear regression of the infection. In contrast, mice immunized with normal mouse IgG, on day 14, six out of six eyes were positive and continued to be positive until day 42. The number of infected eyes began to fall by day 28 seen in this group is believed to be a natural recovery process. In a final experiment, the mice with completely cleared infection from the above experiment were rechallenged with EBs in order to evaluate whether the immunity was associated with memory. A significant factor in terms of protection is the ability of the immunized mice to clear the organisms from their eyes much earlier than the non-immunized ones. This is clearly shown to be the case with mice immunized with monoclonal GLXA-Ab$_2$ (Figure 16 (B)). This shows that monoclonal GLXA Ab$_2$ not only is able to evoke a

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protective immune response but also a memory immune response.

This example illustrates that an anti-idiotypic antibody which mimics GLXA, protects mice as an animal model from chlamydial ocular infection. The mono-clonal antibody mAb$_1$ (89MS30) which was used to produce anti-idiotypic an-tibodies was produced by immunization with whole EBs and screened for its reaction to a genus specific antigen. It has been used to identify GLXA and demonstrated specific binding to the polysaccharide portion of GLXA. This antibody was also found cross-reactive to cLPS.

First GLXA and cLPS are distinctly different genus specific antigens. GLXA was found on RBs, EBs, inclusion membranes, host cell membranes and shed into the inclusion space, cytoplasm of the infected cells and into the surroundings. It was obtained from the supernatant of infected cell culture. Whereas cLPS was found both on EBs and RBs (mainly RBs). They are not secreted or shed from infected cells, but loosely bound to the RBs. Structurally, they have different polysaccharide moieties. GLXA has a unique sugar residue: gulose or gulose derivatives, mannose and galactose, probably arranged in repeating units of guluoronic and mannuronic acids. Only two fatty acids were found associated with the antigen compared to at least 12 in cLPS. Whereas cLPS has typical linear 2-keto-3-dexyoctonoic acid (KDO) trisaccharide (common core) and polysaccharides like glucosamiane and heptose. Serologically, monoclonal GLXA-Ab$_1$ specifically bind to gulose and mannose repeating block. Whereas, cLPS genus specific epitope is on KDO, the specific determinant is the 2-8 linkage. Obviously, it is very unlikely that GLXA and cLPS share the same epitope. It is known that antibodies, whether poly-or monoclonal, antibodies produced either by whole EBs or by purified cLPS which are specific to cLPS have no neutralizing or protective functions. From the composition analysis, it is suggested that gulose together with mannose and galactose form the specific epitope of GLXA. Whereas, cLPS, in addition to KDO trisaccharide as an epitope, has other epitopes in KDO region and also other saccharide portions. These later structures have shown a broad cross-

reaction with LPS from other gram negative bacteria. Since the protective epitope of GLXA consists of an array of sugar residue, it is more reasonable to believe that some of which cLPS is partially shared with GLXA.

There are some other possibilities about this cross-reaction. For example, (1) GLXA and cLPS do not share any primary similarity, but structurally form similar binding motif; (2) although monoclonal GLXA-Ab$_1$ binds to cLPS, GLXA and cLPS do not have the similarity in antibody binding site. The monoclonal antibody can be multispecific, that is, it recognizes a quite different epitope.

From the discussion above, it is believed that monoclonal GLXA-Ab$_1$ is specific to GLXA epitope, the possibility that GLXA and cLPS share same sugar residues or merely structure similarity may explain the cross-reactivity.

Identification of Receptor on Host Cells by Monoclonal GLXA-Ab$_2$ IgG

FACS analysis of the specific binding of monoclonal GLXA-Ab$_2$ to HECEC cells

Human endometrial gland epithelial cells (HECEC) were grown in a 75 mm^2 flask at 37° C with 5% CO_2. When confluent, cells were scraped off the flask using a cell scraper (Baxter, IL) and centrifuged 200 x g for 5 minutes. The cells were rinsed once with 20% FBS in Hanks buffer (Whittker, MD) and passed through a 19 G syringe needle four times to obtain single cells. Serial dilutions of biotin labeled monoclonal GLXA-Ab$_2$ or biotin labeled normal mouse IgG in Hanks buffer, (100 ul) were added to each vial which contained approximately 1.5 x 10^6 HECEC cells and incubated on ice for 30 minutes. Each vial was rinsed twice with 0.02% azide in Hanks buffer. Later, 100 ul of FITC conjugated streptavidin was added and incubated for 30 minutes on ice. After washing twice, the cells were kept in 400 ul of sheath buffer on ice. Cells plus FITC conjugated streptavidin and cells alone were used as background control. Single color flow cytometry was performed immediately by FACS scan (Becton Dickinson).

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Anti-idiotypic antibodies, GLXA-Ab$_3$ and monoclonal GLXA-Ab$_2$

Monoclonal GLXA-Ab$_1$ was injected into guinea pigs subcutaneously in the absence of conjugate or Freund's adjuvant. All four immunized guinea pigs developed high titered anti-idiotypic antibodies specific to monoclonal Ab$_1$, which were found as early as 3 weeks after the first immunization. The titer was approximately 1:5000 by ELISA. The production of the anti-idiotypic antisera contain a relatively high concentration of GLXA-Ab$_2$ which is specific to the hypervariable region of monoclonal GLXA-Ab$_1$. This was shown after two absorptions by normal mouse IgG. When the IgG-1 isotype from guinea pig anti-idiotypic antibodies was used as an immunogen in three rabbits, the titer of anti-anti-idiotypic antibody was more than 1:20,000 two months after immunization. This demonstrates that immunoglobulin itself is a very potent immunogen. This is true not only for interspecies immunization, but also syngeneic immunization. With monoclonal anti-idiotypic antibody monoclonal GLXA-Ab$_2$, the immunization was carried out in syngeneic mice without KLH-conjugation or Freund's adjuvant. The mice developed high titered GLXA-Ab$_3$ in a short time after immunization (9 days). The protocol used in this study is different from most methods which use either a conjugate or Freund's adjuvant for a higher immunogenicity.

A successful vaccine not only requires that it be a good immunogen but that it is long lasting (preferably for the lifetime off the host). The immunity produced by idiotype is long lived. The ability to inhibit the binding of monclonal GLXA-Ab$_1$ to GLXA by guinea pig GLXA-Ab$_2$ from three immunized guinea pigs have been monitored for as long as 77 weeks. With only three boosts, the inhibition one year post immunization is almost equal to antisera collected in the early stages after the immunization. This indicates that the immunity elicited by the idiotypic antibody monoclonal GLXA-Ab$_1$ has a long term memory. Since the half-life of an antibody molecule or the majority of antibody-producing cells is about a few weeks, the boosting interval (six months) is far beyond the life span of the B cells and the immunoglobulins. It is the constant stimulation within the idiotypic network that

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keeps this anti-idiotypic antibody at a certain level. The change of idiotypic specificity during this period has not been seen in this case.

An internal image of chlamydial GLXA, isotypic difference

In this study, guinea pig GLXA-Ab$_2$ IgG-1 and IgG-2 were separated. The regulatory function of these two isotypes to the idiotype was not evaluated. However, the difference between IgG-1 and IgG-2 subclasses have been found in inhibition of the binding of monoclonal GLXA-Ab$_1$ to GLXA. With a novel system, chemiluminometric immune-assay, the incubation and the final detection were all carried out in solution rather than solid phase as in ELISA, thus greatly lessening the possibility of the inhibition by hindrance. The results have shown that GLXA-Ab$_2$ IgG-1 inhibited 100% of the binding whereas IgG2 50% at the same concentration. This suggested that GLXA-Ab$_2$ IgG-1 has a high affinity in binding to the idiotype or being more like the antigen, GLXA. This demonstrates an isotypic difference in their binding ability to the idiotype which reflects a difference in their respective active sites. IgG-1 has a different idiotype binding ability from IgG1. There are a number of examples of dominant idiotypes, for example, A5A idiotope of anti-strep-A carbohydrate antibodies or the T15 idiotope of phosphoryl choline antibodies. It is not clear if there is any isotype preference of anti-idiotypic antibody in different systems. This finding suggests that a certain isotype of GLXA-Ab$_2$ is the internal image while others are not.

Monoclonal GLXA-Ab$_2$ as an immunogen of chlamydial GLXA

The purpose of making monoclonal anti-idiotypic antibodies is to: (1) have a constant source of anti-idiotypic antibody for vaccine study; (2) identify a possible receptor for GLXA on host cells; and (3) further characterize the epitope on GLXA. This enables an understanding of biological functions of GLXA in terms of epitope

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density, its role in mechanism of infection and the protective function against chlamydial infection in vivo. The production of monoclonal anti-idiotypic antibodies was carried out in the syngeneic BALB/cBYJ mice. In the first fusion, one stable, highly inhibitory clone (91MS441) from 283 clones screened was selected. In the second fusion, another clone (91MS442) was selected though it is not as inhibitory as 91MS441 clone in chemiluminometric immunoassay. The monoclonal GLXA-Ab_2 produced by this clone (91MS441) has been shown to be the internal image of the chlamydial antigen, GLXA.

It is interesting to note that the inhibitory ability of mouse GLXA-Ab_3 slightly but obviously decreases over time. The dosage of the anti-idiotype has been a factor in either enhancing the idiotype or suppressing the idiotype. This inhibition results by GLXA-Ab_3 has shown that after administration of monoclonal GLXA-Ab_2 IgG twice, the inhibition is higher than the sera obtained after administration three times. This indicated 50 ug is either too much for one dose or too much for repeated administrations. On the other hand, it shows that a low amount is enough for protective immunity. The reason for choosing normal mouse IgG as a negative control in the immunization rather than a non-relative clone is that it would prevent any possible bias from a specific clone.

Monoclonal GLXA-Ab_1 and GLXA-Ab_3 bear the same antigen binding structure.

GLXA-Ab_3 from immunized rabbits and mice recognized affinity purified GLXA by immune-dot blot assay, though there is no previous exposure to GLXA or infection. The binding of monoclonal GLXA-Ab_1 to GLXA is inhibited as the concentration of GLXA-Ab_3 increases. This indicates that the anti-anti-idiotype has equivalent antigen reactivity as idiotype, that is they recognize the same epitope. The same antigen reactivity of monoclonal GLXA-Ab_1 and GLXA-Ab_3 from rabbits to GLXA was further proved in the immune-fluorescent staining of the inclusions in infected McCoy cell culture. The experiment suggested the structural similarity of antibodies. Using monoclonal GLXA-Ab_2 which mimic GLXA to identify its role

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would be valuable in understanding the mechanism of anti-idiotype protection. The experiment was carried out with human epidermoid carcinoma cells (A431) as well as human endometrial gland epithelial cells (HEGEC). Those cell lines were used because of their human origin. A preliminary binding experiment was performed by direct antibody staining detected with FACScan (Becton Dickinson, N.J.) flow cytometer. Since the separation of these epithelial cells into a single cell suspension is very difficult without using enzyme or harsh separation, especially HEGEC cells, the cell population consists of singlets, doublets and multiplets. Single cell population was gated right above the cell debris. Since the control, cells stained with normal mouse IgG was at exactly the same gate, it is believed that the two groups are comparable. In addition, the binding is direct, monoclonal GLXA-Ab$_2$ or normal mouse IgG was directly biotin labeled. The intensity of monoclonal GLXA-Ab$_2$ bound increases with the concentration specifically to the host cells (HEGEC). While normal mouse IgG has no such binding even at the highest concentration. If this finding can be repeated and proved to be valid, GLXA is an adhesion or a ligand which binds to HEGEC cells. There are some reasons to believe that GLXA is an adhesion or a ligand. First, as mentioned above, GLXA-Ab$_3$ neutralization occurs at the very early stage. This is proved by finding apparently no significant chlamydial specific ribosomal RNA in conjunctiva samples taken from primates inoculated with GLXA-Ab$_3$ treated EBs. This suggested that GLXA-Ab$_3$ blocked EBs from getting into the host. Second, GLXA was found to enhance the infection by approximately three fold when McCoy cells were pre-incubated with GLXA. It is only if GLXA as an adhesion or ligand which facilitate the attachment of EBs to host cells that this result can occur. Actually, EBs may well use this mechanism to infect host cells because GLXA are secreted from infected cells.

A possible mechanism of the role of GLXA may be the following: EBs attach to the host cells either by GLXA on EBs or by free GLXA which were secreted by the infected cells into the surroundings. This makes it much easier and efficient for GLXA to absorb to surrounding cells. Ebs can then attach to the host cells

either by GLXA on EBs, thus binding to the host cells. This mechanism seems much more efficient than one to one attachment.

Neutralization of chlamydial infection by GLXA-Ab₃

The preliminary neutralization test in vitro has shown that more chlamydial inclusions were found with GLXA-Ab₁ treated than rabbit Ab₃ treated C. trachoma- tis serovar B EBs. This showed that GLXA-Ab₃ neutralized the infection, whereas monoclonal GLXA-Ab₁ did not. GLXA-Ab₃ neutralized the infection in cell culture whereas monoclonal GLXA-Ab₁ did not. This in vitro result was repeated in vivo in two later experiments with the primate infection model. It was confirmed that GLXA-Ab₃ effectively neutralized chlamydial infection both in vitro and in vivo.

The understanding of the mechanism of this neutralization comes from the chlamydia specific RNA hybridization experiment. The primates were ocularly inoculated with GLXA Ab₃ or normal rabbit IgG treated EBs. Chlamydial RNA was detected from the conjunctival swabs taken from primates on different days prior and post inoculation. The RNA hybridization assay used in this study is an extremely sensitive way to detect chlamydial infection in samples which are unequivocally negative by either cell culture of DFA or both. The substantially low RNA found in Ab₃ treated primates provides the evidence that the neutralization occurs at very early stage of the infection, before the internalization of the or- ganisms. This suggests that GLXA may be an adhesion or ligand functioning at the stage of attachment.

The role of humoral immunity in protection against chlamydial infection has long been discrepant. However, it has been shown both in vitro and in vivo that humoral immunity may play some role in preventing chlamydial infection.

Protection of Mice from chlamydial infection by immunization with monoclonal GLXA-Ab₂

The chlamydial ocular infection model in BALB/cByJ mouse was employed

in a vaccine study with monoclonal anti-idiotypic antibody monoclonal GLXA-IgG. It is model for C. trachomatous serovars which only infect humans. In the immunization experiments, monoclonal (GLXA-Ab$_2$) IgG (50 ug/mouse) was used to immunize 6 to 20 mice in each group subcutaneously either in the presence of adjuvant or with no adjuvant. Mice immunized with monoclonal GLXA-Ab$_2$ mount a distinctive GLXA-Ab$_3$ titer detectable one week after a single immunization. The system used in this study is syngeneic: monoclonal GLXA-Ab$_1$, monoclonal GLXA-Ab$_2$ or GLXA-Ab$_3$ are all produced in the same strain of mice. They bear the same immunoglobulins genetically. The only "foreign structure" is the specific binding site of each type antibody (monoclonal GLXA-Ab$_1$ or monoclonal GLXA-Ab$_2$). Normal mouse IgG from the same strain was used as a negative control. Consistently, mice immunized with monoclonal GLXA-Ab$_2$ developed a significantly high GLXA-Ab$_3$ titer against purified GLXA compared to the control group. The average titer was as high as 1:3200 comparing with 1:200 in the mice immunized with normal mouse IgG. This clearly demonstrates that the production of GLXA-Ab$_3$ antibody in synergenic mice is elicted by the hypervariable region, the structure which mimics the GLXA. In addition, the GLXA-Ab$_3$ from these mice inhibited the binding of monoclonal GLXA-Ab$_1$ to GLXA as efficiently as unlabeled monoclonal GLXA-Ab$_1$. These experiments shows that monoclonal GLXA-Ab$_2$ mimics one single epitope of GLXA.

The protection of mice from ocular infection by C. trachomatous was consistently demonstrated in four individual experiments in vivo. The mice immunized with monoclonal GLXA-Ab$_2$ or normal mouse IgG (three times on a weekly basis) were infected in the eyes by inoculation of EBs (5000 IFU/eye). The conjunctival swabs were taken on different days before and after the infection. Inclusions were detected by cell culture. The eyes of the mice immunized with monoclonal GLXA-Ab$_2$ IgG have significantly short recovery (9 days after ocular inoculation comparing to 28 days of the control immunized with normal mouse IgG). The GLXA-Ab$_3$ titer is well correlated with cell culture results. This demon-

strates that monoclonal GLXA-Ab$_2$ which mimics a single GLXA epitope, is an effective immunogen, and evokes a strong protective immune response against chlamydial infection. This shows that a single protective epitope resides on chlamydial GLXA.

Prospective of the mechanism of the neutralization and protection

This example is the first demonstration that a non-neutralizing monoclonal GLXA-Ab$_1$ can produce a neutralizing GLXA-Ab$_3$ in vivo through an anti-idiotypic antibody (GLXA-Ab$_2$). The central issue is that monoclonal GLXA-Ab$_1$ produced by immunization with chlamydial EBs does not neutralize or protect primates from reinfection whereas GLXA Ab$_3$ produced by guinea pig GLXA-Ab$_2$ neutralized the infection and immunization with monoclonal GLXA-Ab$_2$ protects mice from reinfection.

Assay results in Examples 2-5 for the presence of GLXA in in vitro and in vitro (including cellular and non-cellular material) samples are as follows:

GLXA has been found to be present in Chlamydia EB suspensions prepared from in vitro cell culture when assayed in Examples 2, 3, 4 and 5.

GLXA has been found to be present in Chlamydia infected in vitro cell culture supernatants when assayed in Examples 2 and 4.

GLXA has been found to be present in cervical swab specimens from cell culture confirmed Chlamydia infected females when assayed in Examples 3,4 and 5.

GLXA has been found to be present in vaginal swab specimens from cell culture confirmed Chlamydia infected females when assayed in Example 5.

GLXA has been found to be present in urine specimens from cell culture confirmed Chlamydia infected females when assayed in Examples 4 and 5.

Example 2

A sandwich ELISA assay.

Polystyrene microliter plate wells are coated with rabbit polyclonal antisera

containing anti-GLXA antibodies (GLXA-Ab$_1$).

Monoclonal anti-GLXA antibody (GLXA-Ab$_1$) is conjugated to horseradish peroxidase (HRP).

Assay Protocol:

1) Pipet 100 ul of sample per well

2) Pipet 100 ul of assay buffer per well.

3) Incubate for one hour @ 37° C.

4) Wash the wells 3X with PBS/tween, blot dry.

5) Pipet 200 ul per well monoclonal anti-GLXA: HRP conjugate.

6) Incubate for one hour @ 37° C.

7) Wash the wells 2X with PBS/tween then 2X with PBS, blot dry.

8) Pipet 200 ul per well substrate/chromogen solution.

9) Incubate for 30 minutes @ room temperature.

10) Stop the reaction by pipetting 100 ul per well 1N H$_2$SO$_4$.

11) Read the absorbance of each well in a spectrophotometer.

<u>Example 3</u>

An amplified sandwich ELISA assay.

Polystyrene microliter plate wells are coated with monoclonal anti-GLXA antibody (GLXA-Ab$_1$). Monoclonal anti-GLXA antibody if biotinylated. Monoclonal anti-Biotin antibody is conjugated to horseradish peroxidase (HRP).

Assay Protocol:

1) Pipet 140 ul of assay buffer per well

2) Pipet 10 ul of sample per well.

3) Incubate for 30 minutes @ room temperature.

4) Wash the wells 3X with PBS/tween, blot dry.

5) Pipet 150 ul per well biotinylated anti-GLXA antibody.

6) Incubate for 30 minutes @ room temperature.

7) Wash the wells 3X with PBS/tween, blot dry.

8) Pipet 150 ul per well anti-biotin: HRP conjugate.

9) Incubate for 30 minutes @ room temperature.

10) Wash the wells 5X with PBS/tween, blot dry.

11) Pipet 150 ul per well substrate/chromogen solution.

12) Incubate for 6 minutes @ room temperature.

13) Stop the reaction by pipetting 100 ul per well 1N H_2SO_4.

14) Read the absorbance of each well in a spectrophotometer.

For the sandwich ELISA assays the final absorbance in each well is directly proportional to the amount of GLXA in the sample. Any absorbance greater than 2X the absorbance of the negative control is indicative of the presence of GLXA in the sample.

Example 4

A competing ELISA assay.

Polystyrene microliter plate are coated with monoclonal anti-GLXA antibody ($GLXA-Ab_1$). Monoclonal anti-idiotypic antibody $GLXA-Ab_2$) is conjugated to horse-radish peroxidase (HRP).

Assay Protocol:

1) Pipet 200 ul of sample per well

2) Incubate for one hour @ 37° C.

3) Pipet 100 ul per well $GLXA-Ab_2$:HRP per well.

4) Incubate for 30 minutes @ room temperature.

5) Wash the wells 4X with PBS/tween, blot dry.

6) Pipet 200 ul of substrate/chromogen per well.

7) Incubate for 20 minutes @ room temperature.

8) Stop the reaction by pipetting 100 ul per well 1N H_2SO_4.

9) Read the absorbance of each well in a spectrophotometer.

Example 5

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An amplified competitive ELISA assay. Polystyrene microtiter plate wells are coated with monoclonal anti-idiotypic antibody (GLXA-Ab$_2$). Monoclonal anti-GLXA (GLXA-Ab$_1$) antibody is biotinylated. Monoclonal anti-biotin is conjugated to horseradish peroxidase (HRP).

1) Pipet 100 ul of assay buffer per well

2) Pipet 30 ul of sample per well

3) Pipet 20 ul of biotinylated GLXA-Ab$_1$ per well.

4) Incubate for 45 minutes @ room temperature.

5) Wash the wells 3X with PBS/tween, blot dry.

6) Pipet 150 ul of anti-biotin: HRP conjugate per well.

7) Incubate for 45 minutes @ room temperature.

8) Wash the wells 5X with PBS/tween, blot dry.

9) Pipet 150 ul per well substrate/chromogen solution.

10) Incubate for 5 minutes @ room temperature.

11) Stop the reaction by pipetting 100 ul per well 1N H$_2$SO$_4$.

12) Read the absorbance of each well in a spectrophotometer.

For the competitive ELISA assays the final absorbance in each well is inversely proportional to the amount of GLXA in the sample. The % inhibition is calculated by the formula:

$$\% \text{ inhibition} = \frac{[(\text{Abs. neg. control}) - (\text{Abs. sample})] \times 100\%}{\text{Abs. neg. control}}$$

Any inhibition greater than 20% is indicative of the presence of GLXA in the sample.

Assay results for the presence of GLXA in _in vitro_ and _in vivo_ (including cellular and non-cellular material) samples are as follows:

GLXA has been found to be present in Chlamydia EB suspensions prepared from _in vitro_ cell culture when assayed in the above example assays #s 2, 3, 4 and 5.

GLXA has been found to be present in Chlamydia infected _in vitro_ cell

culture supernatants when assayed in the above example assays #s 2 and 4.

GLXA has been found to be present in cervical swab specimens from cell culture confirmed Chlamydia infected females when assayed in the above example assays #s 3, 4 and 5.

GLXA has been found to be present in vaginal swab specimens from cell culture confirmed Chlamydia infected females when assayed in the above example assays # 5.

GLXA has been found to be present in urine specimens from cell culture confirmed Chlamydia infected females when assayed in the above example assays #s 4 and 5.

CLAIMS

1. The method for the immunochemical diagnosis of Chlamydia in a mammal which comprises contacting an extracellular biological fluid from said mammal with antibody specific for an antigenic determinant of Chlamydial glycolipidexoanatigen from substantially any Chlamydia species or serovar and determining extent of binding of said antibody with said antigenic determinant.

2. The method according to claim 1 which comprises measuring the amount of material immunologically bound by said antibody and determining by comparing, for a positive diagnosis, whether the amount of bound material is greater than that found with specimen from an uninfected mammal.

3. The method according to claim 1 which comprises determining the amount of glycolipidexoantigen in the specimen via a competitive immunoassay utilizing an antiidiotypic antibody that contains an internal image of the specific epitope of Chlamydial glycolipidexoantigen.

4. The method according to claim 1 wherein the immunochemical diagnoses method is selected from the group consisting of radioimmunoassay, enzyme-linked immunosorbent assay, fluorescent assay, chemiluminescent assay, bioluminescent assay, competitive immunoassay, membrane-based immunoassay, precipitation, agglutination and antigen capture.

5. The method according to claim 1 wherein the antibody specific for Chlamydia glycolipidexoantigen is monoclonal.

6. The method according to claim 1 wherein the antibody specific for Chlamydia glycolipidexoantigen is polyclonal.

7. The method according to claim 3 wherein the anti-idiotype antibody that contains the internal image of the specific epitope of Chlamydial glycolipidexo-antigen is monoclonal.

8. The method according to claim 3 wherein the anti-idiotype antibody that contains the internal image of the specific epitope of Chlamydial glycolipidexo-antigen is polyclonal.

9. The method according to claim 1 wherein the antibody specific for Chlamydial glycolipidexoantigen is an anti-anti-idiotypic antibody of claim 7 as an immunogen.

10. The method according to claim 9 wherein the anti-anti-idiotypic antibody is polyclonal.

11. The method according to claim 9 wherein the anti-anti-idiotypic antibody is monoclonal.

FIG. 1

FIG. 2

2/9

Reciprocal Dilution of Antibody

FIG. 3

Fraction (0.5 ml)

FIG. 4

3/9
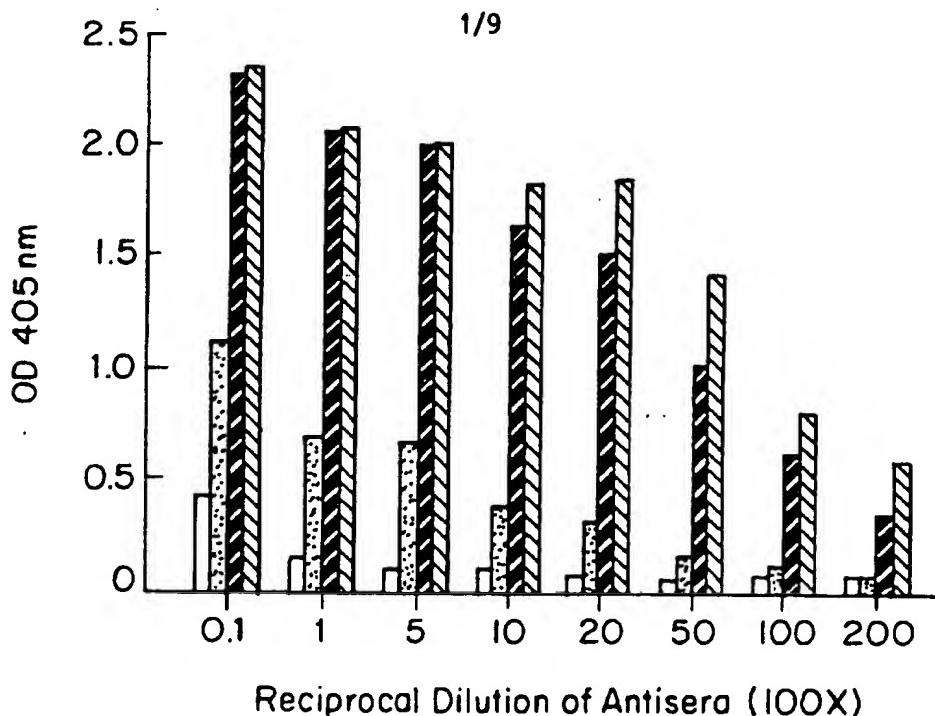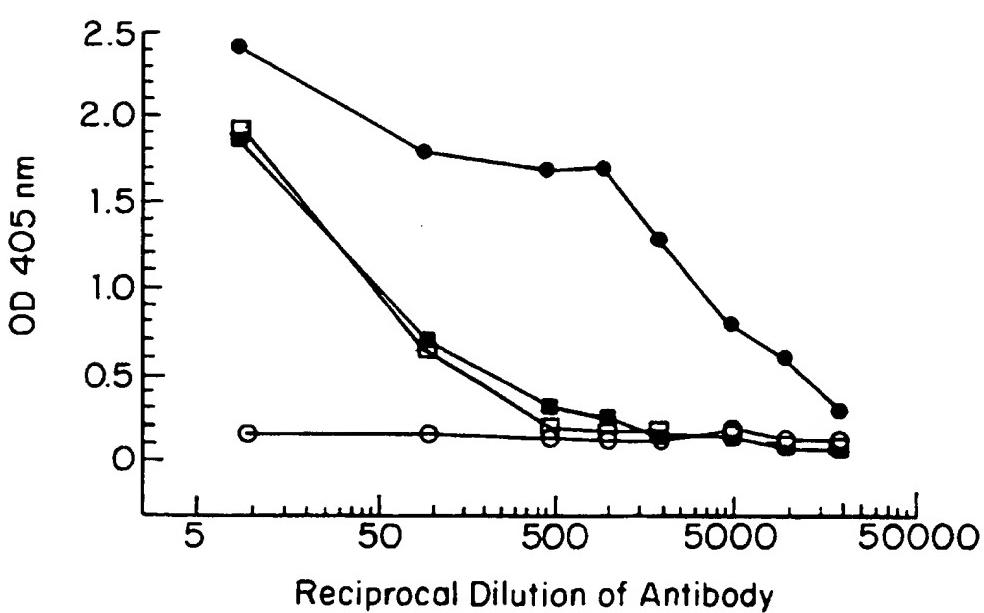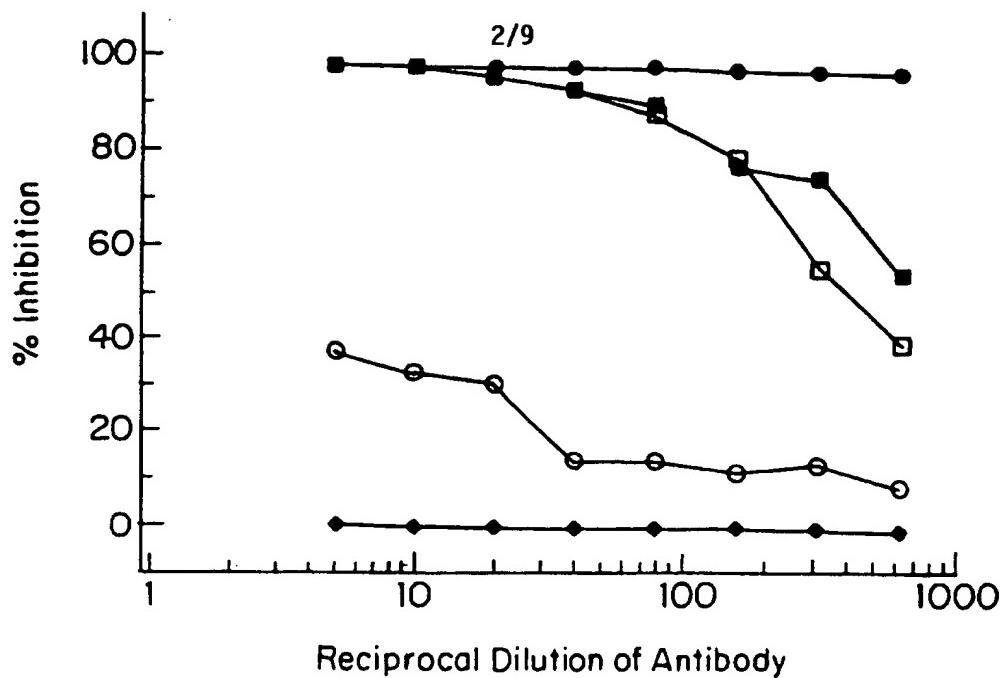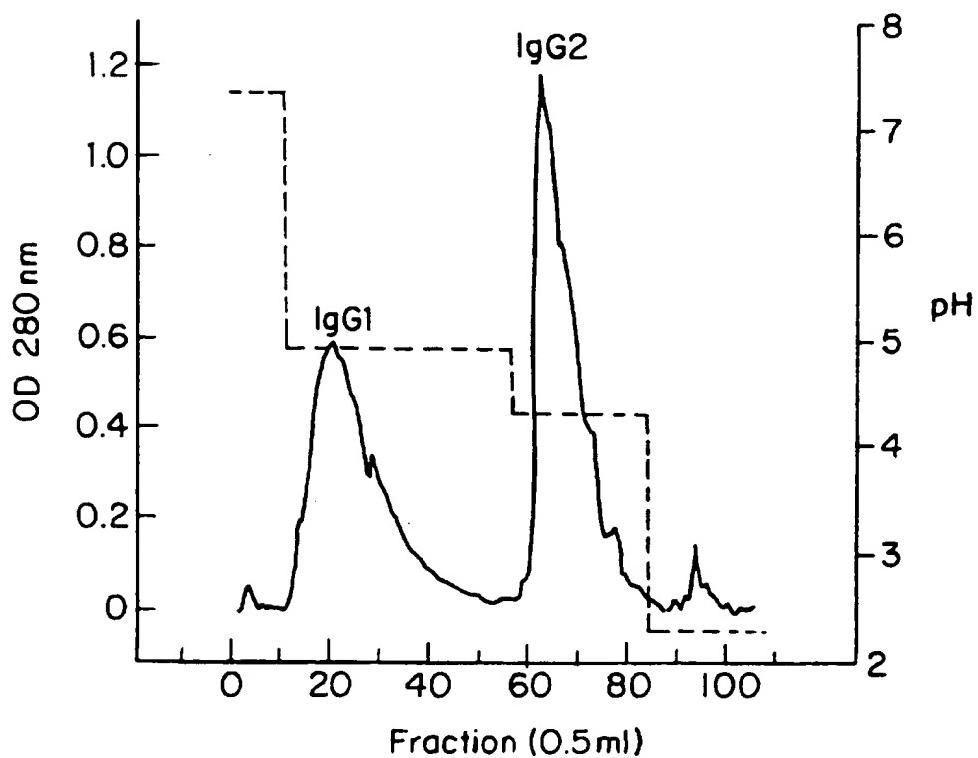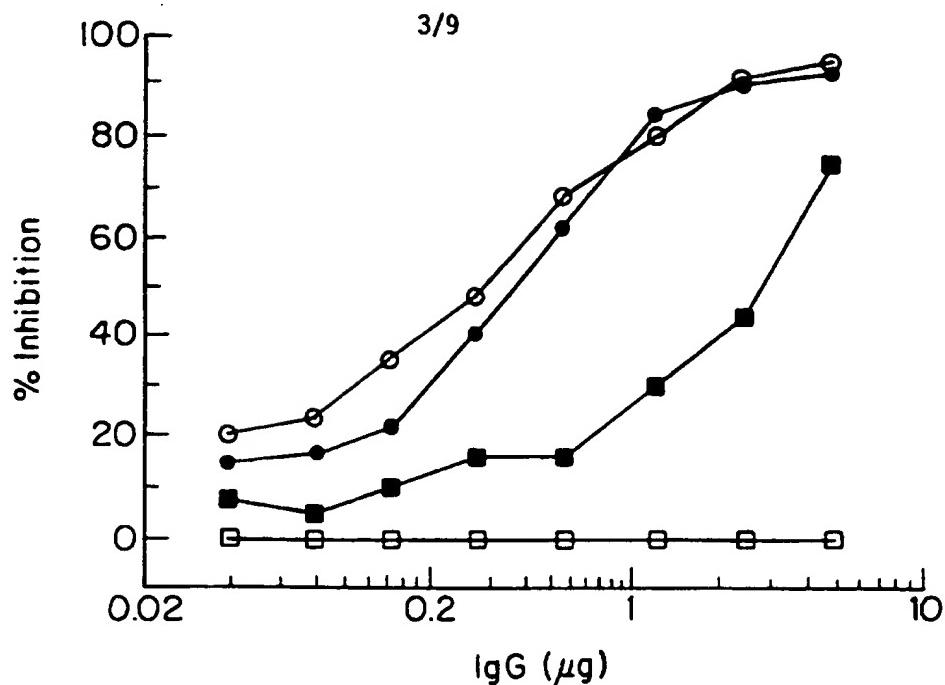
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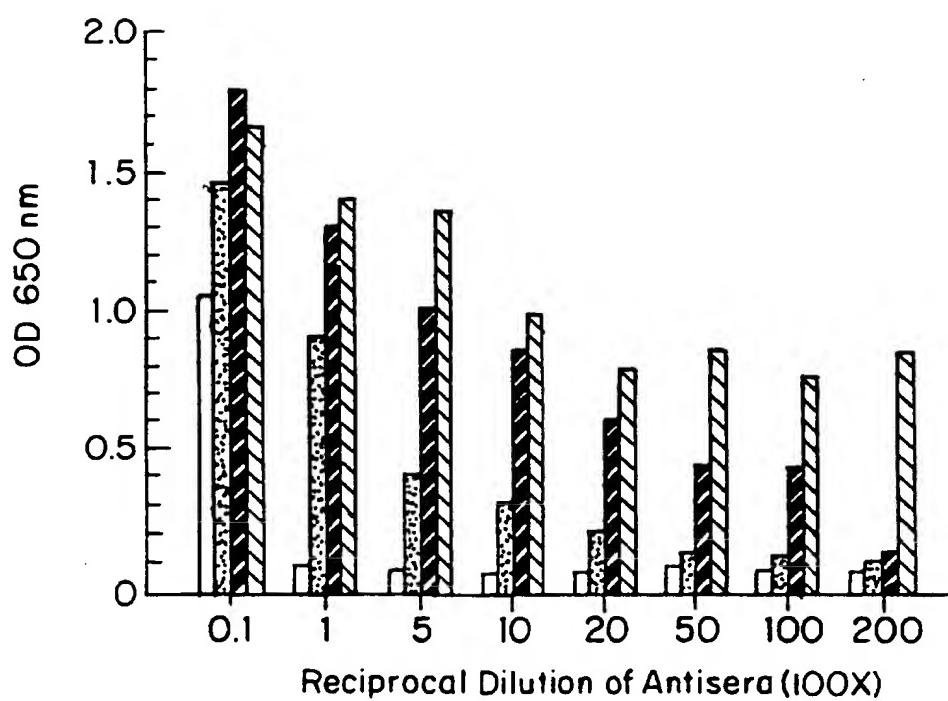
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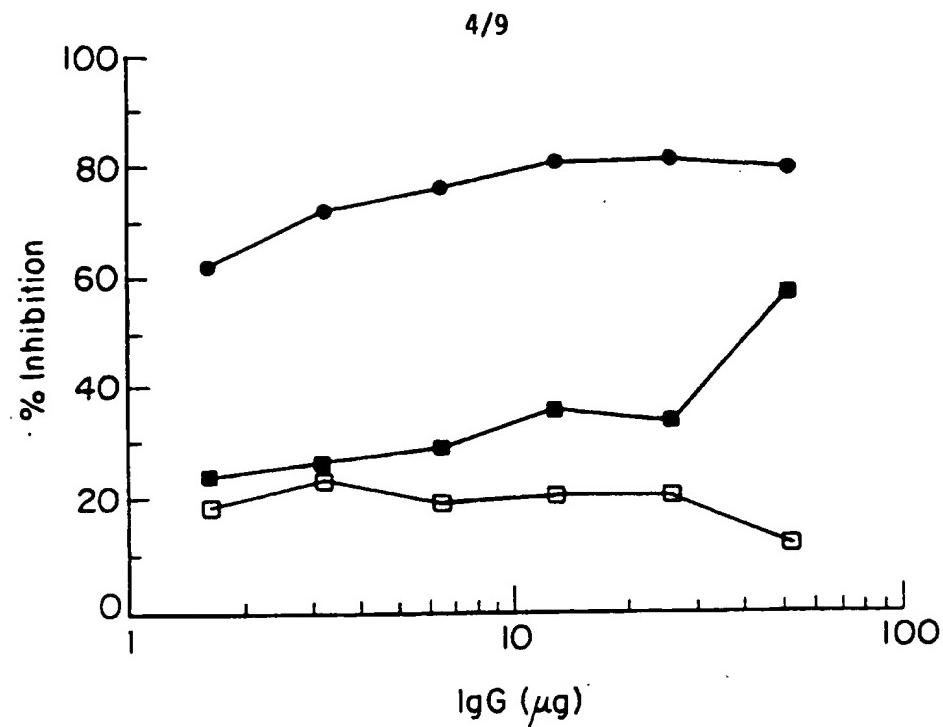
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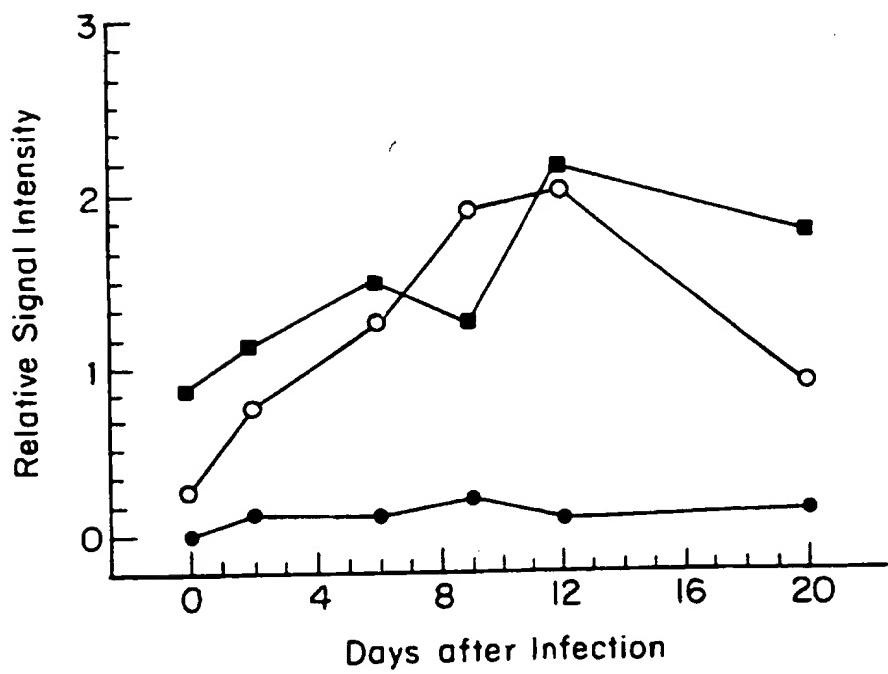
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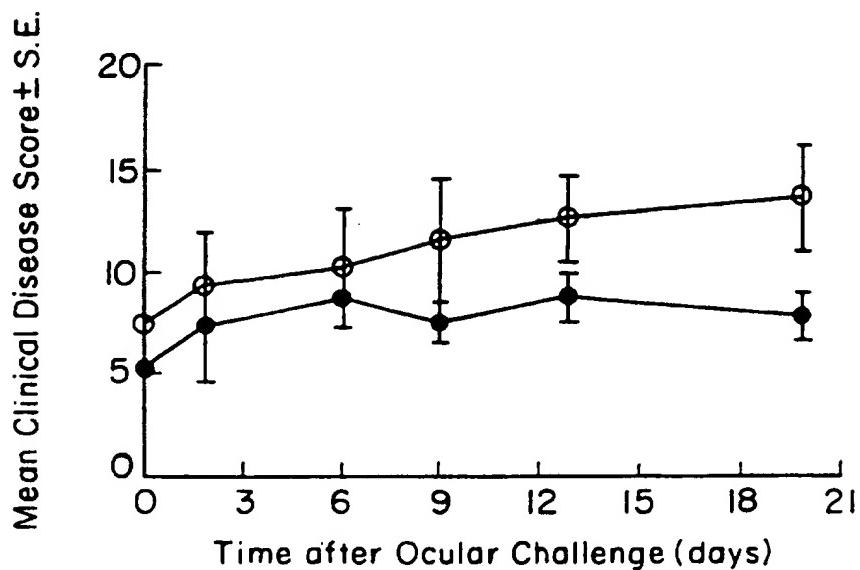
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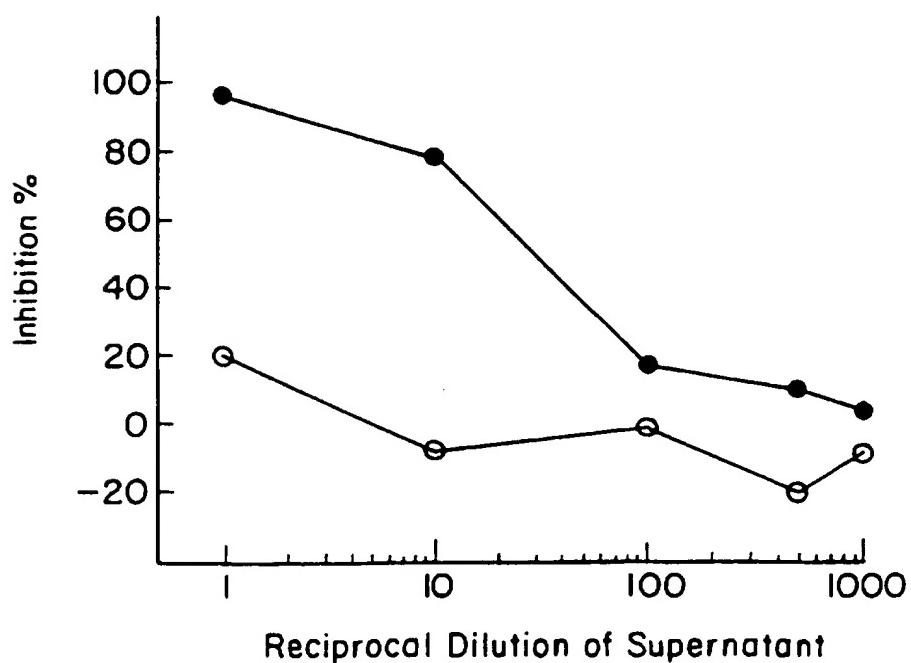

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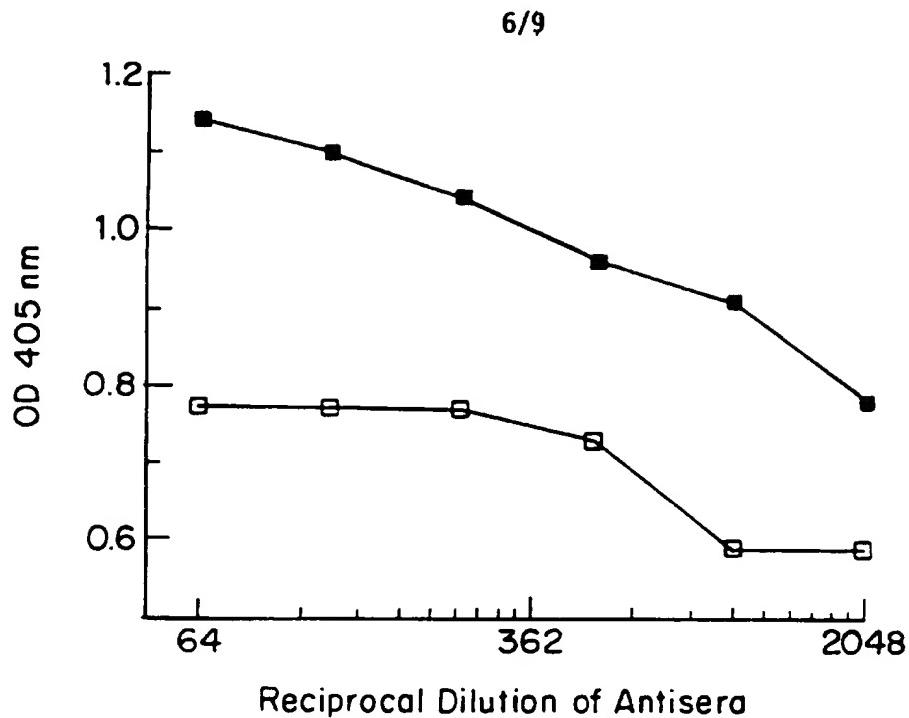
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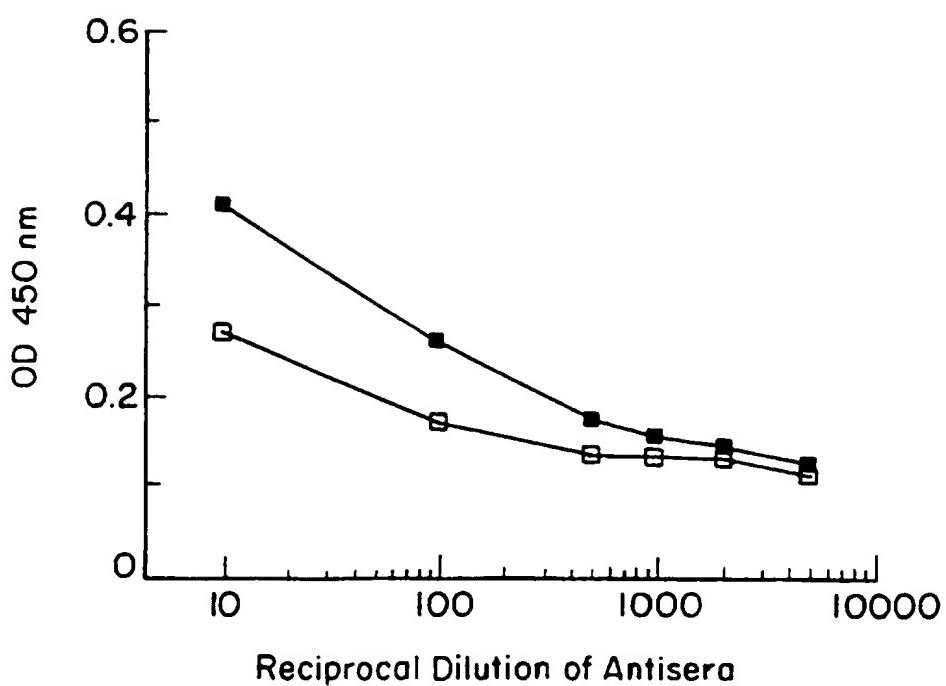
FIG. 12
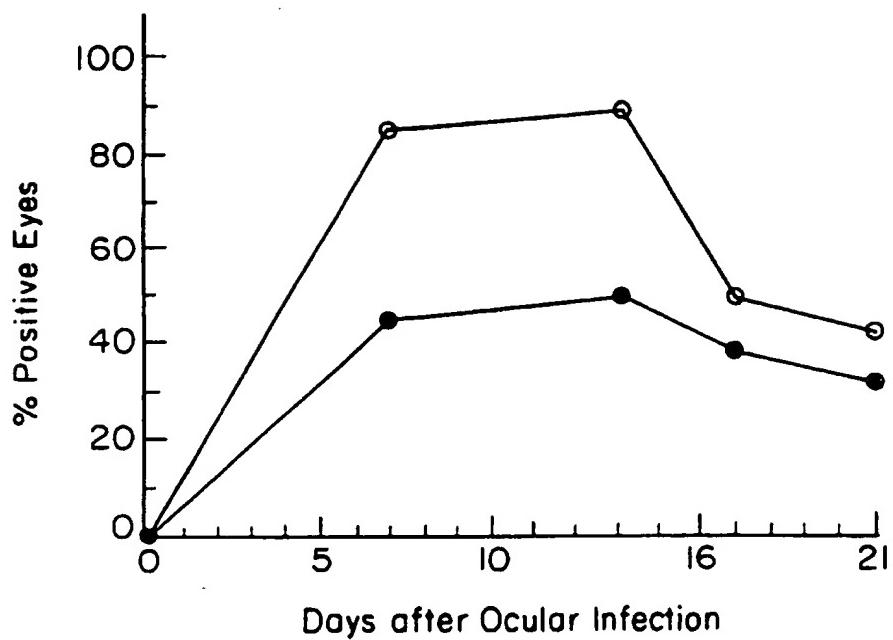
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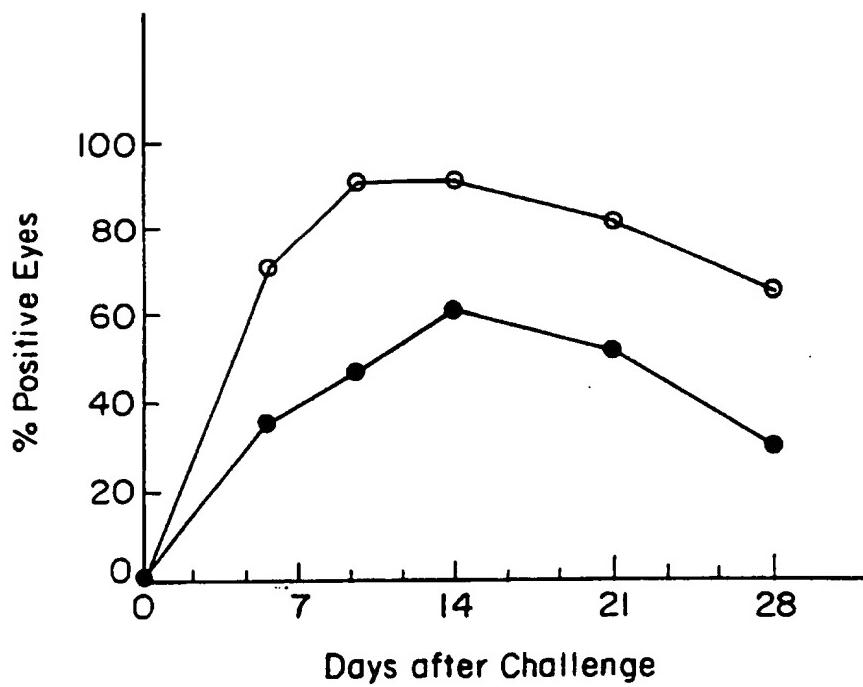
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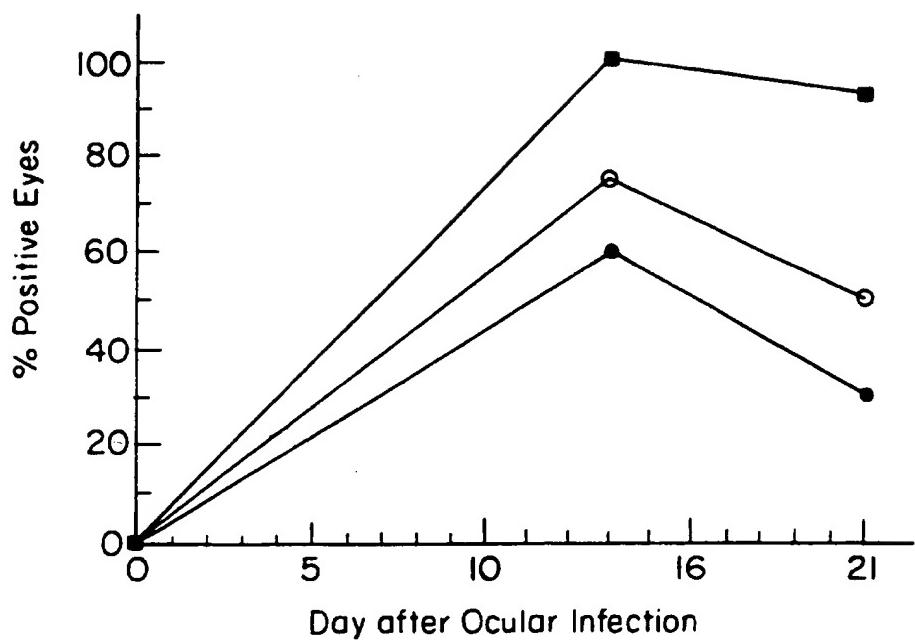
FIG. 15
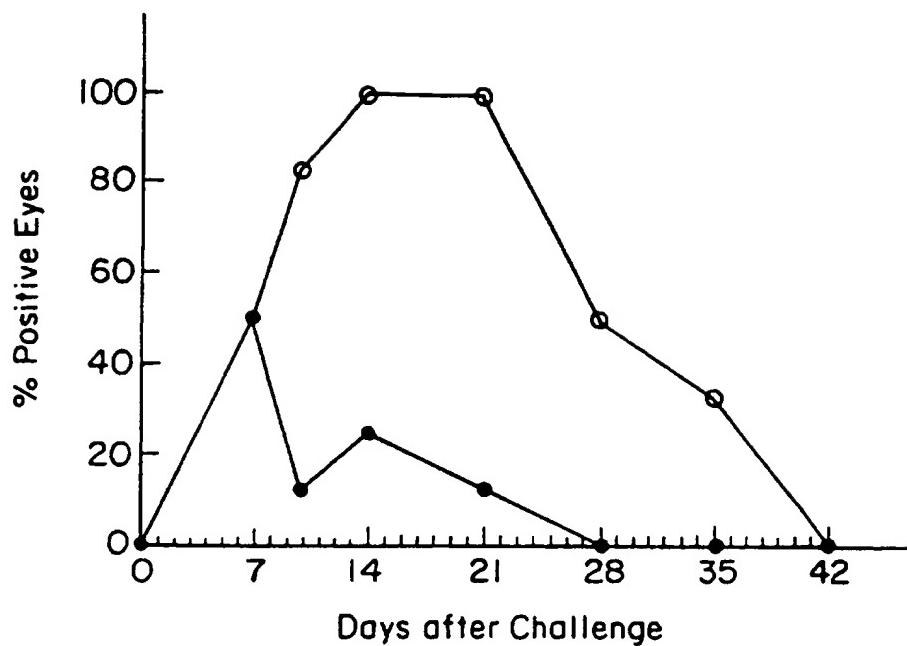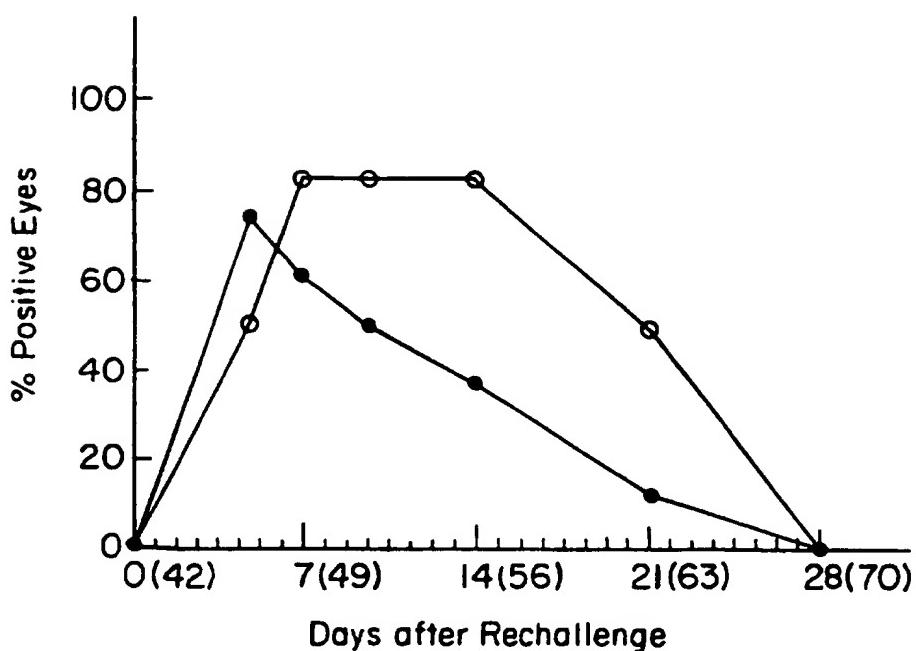
FIG. 16(A)

FIG. 16 (B)

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 33/569
US CL   :435/7.32, 7.36, 7.92, 7.93; 436/518, 536, 547, 548

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.32, 7.36, 7.92, 7.93; 436/518, 536, 547, 548

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, WPIDS
search terms: chlamydia?, antibod?, glycolipi?, idiotyp?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,085,986 A (MAUCK ET AL) 04 FEBRUARY 1992, column 3, lines 13-52.	1-2, 4-6
X	US 5,234,817 A (PRONOVOST ET AL) 10 AUGUST 1993, column 3, lines 4-45 and column 4, lines 23-25.	1-2, 4-6
Y	BLANCHARD et al. 'Internal image of exoglycolipid genus specific antigen produced by anti-idiotype.' In: 7th International Symposium on Chlamydial Infections. Edited by W.R. Bowie. Cambridge: Cambridge University Press, 1990, pages 205-208, see entire document.	1-8

[X] Further documents are listed in the continuation of Box C.   [ ] See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	
"E" earlier document published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 MAY 1996	1 3 JUN 1996

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	GARY TANIGAWA
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/02617

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	STUART et al. Some characteristics of a secreted chlamydial antigen recognized by IgG from *C. trachomatis* patient sera. Immunology. 1989, Vol. 68, pages 469-473, especially page 472.	1-8
Y	KENNEDY et al. Vaccines utilizing internal image anti-idiotypic antibodies that mimic antigens of infectious organisms. BioTechniques. 1985, Vol. 3, No. 5, pages 404-406 and 408, especially pages 404-405.	7

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/02617

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 9-11
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)*